DEVELOPMENT OF CONVENTIONAL AND TAQMAN REAL-TIME POLYMERASE CHAIN REACTION METHODS FOR DETECTION OF LIVER FLUKE INFECTION WITH *PLATYNOSOMUM* SPP. IN CAT FECES

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การพัฒนาวิธีการตรวจหาการติดพยาธิใบไม้ในตับ *Platynosomum* spp. จากมูลแมวด้วยวิธีปฏิกิริยาลูกโซ่แบบดั้งเดิมและแบบใช้ TaqMan

นายเชน ธีฮา โซ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DEVELOPMENT OF CONVENTIONAL AND TAQMAN REAL-TIME POLYMERASE CHAIN REACTION METHODS FOR DETECTION OF LIVER FLUKE INFECTION WITH *PLATYNOSOMUM* SPP. IN CAT FECES
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เชน ธีฮา โซ : การพัฒนาวิธีการตรวจหาการติดพยาธิใบไม้ในตับ *Platynosomum* spp. จากมูลแมวด้วยวิธีปฏิกิริยาลูกโซ่แบบดั้งเดิมและแบบใช้ TaqMan. (DEVELOPMENT OF CONVENTIONAL AND TAQMAN REAL-TIME POLYMERASE CHAIN REACTION METHODS FOR DETECTION OF LIVER FLUKE INFECTION WITH *PLATYNOSOMUM* SPP. IN CAT FECES) อ.ที่ปรึกษาหลัก : วรพร สุขุมาวาสี, อ.ที่ปรึกษาร่วม : อรวรรณ พู่พิสุทธิ์

พยาธิใบไม้แพลททีโนโซมุม (*Platynosomum* spp.) เป็นสาเหตุของโรค platynosomiasis ที่ทำให้แมวเป็นโรคตับและท่อน้ำดี การตรวจมูลโดยใช้เทคนิคการปั่นเหวี่ยงให้ตกตะกอนถือเป็นวิธีมาตรฐานหลักในการตรวจหาไข่พยาธิ *Platynosomum* spp. อย่างไรก็ตาม การตรวจหาเชื้อภายใต้กล้องจุลทรรศน์นั้นมีข้อจำกัดในด้านความไว โดยเฉพาะในกรณีที่มีการอุดตันของท่อน้ำดีอย่างสมบูรณ์ รวมทั้งการปล่อยไข่พยาธิอย่างไม่สม่ำเสมอ เพื่อแก้ปัญหาดังกล่าว การศึกษานี้มีเป้าหมายเพื่อพัฒนาวิธีทางอณูชีววิทยาเพื่อตรวจหาการติดเชื้อ *Platynosomum* spp. จากมูลของแมว โดยใช้วิธีปฏิกิริยาลูกโซ่โพลิเมอเรสแบบดั้งเดิม (cPCR) และวิธีปฏิกิริยาลูกโซ่โพลิเมอเรสแบบแท็คแมนเรียลไทม์ (rtPCR) ซึ่งมีเป้าหมายที่ตำแหน่งยีนส์ไอทีเอส 1 (ITS1) จากตัวอย่างมูลแมวทั้งสิ้น 120 ตัวอย่าง ผลการศึกษาพบว่าเมื่อเปรียบเทียบกับการตรวจหาเชื้อภายใต้กล้องจุลทรรศน์ วิธี cPCR มีค่าความไว ค่าความจำเพาะ ค่าทำนายเมื่อผลเป็นบวก (PPV) และค่าทำนายเมื่อผลเป็นลบ (NPV) เท่ากับร้อยละ 98.3 ร้อยละ 88.3 ร้อยละ 89.4 และ ร้อยละ 98.2 ตามลำดับ นอกจากนี้ วิธี rtPCR สามารถตรวจพบความชุกของการติดเชื้อได้สูง (ร้อยละ 55; 66/120) กว่าวิธีมาตรฐาน (ร้อยละ 50; 60/120) และวิธี cPCR (ร้อยละ 45.8; 55/120) วิธี rtPCR ที่ถูกพัฒนาในการศึกษานี้แสดงให้เห็นถึงค่าดัชนีดังกล่าวมีค่าสูงในระดับที่ยอมรับได้ จึงมีความเป็นไปได้ที่จะใช้วิธีเหล่านี้ในการเพิ่มโอกาสการวินิจฉัยการติดเชื้อ *Platynosomum* spp. ในแมว ที่อาจได้ผลลบลวงจากวิธีมาตรฐาน

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Shane Thiha Soe : DEVELOPMENT OF CONVENTIONAL AND TAQMAN REAL-TIME POLYMERASE CHAIN REACTION METHODS FOR DETECTION OF LIVER FLUKE INFECTION WITH *PLATYNOSOMUM* SPP. IN CAT FECES. Advisor: Asst. Prof. Dr. WORAPORN SUKHUMAVASI Co-advisor: Dr. Orawan Phuphisut

The *Platynosomum* spp. fluke is the etiologic agent of platynosomiasis responsible for hepatobiliary diseases in cats. Coprological examination using centrifugal sedimentation technique is the gold standard method for detection of *Platynosomum* spp.'s eggs. However, microscopic examination method has a limited sensitivity, particularly in cases of complete biliary obstruction and inconsistent egg shedding. To circumvent these problems, this study aims to develop molecular-based methods for detecting *Platynosomum* spp. infection in cat feces including conventional PCR (cPCR) and TaqMan real-time PCR methods targeting ITS1 region. From a total number of 120 cat fecal samples, when compared to microscopic examination, the cPCR method had sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 80.0%, 88.3%, 87.3%, and 81.5%, respectively, while the TaqMan real-time PCR method had these values of 98.3%, 88.3%, 89.4%, and 98.2%, respectively. Furthermore, the TaqMan real-time PCR can assess a greater prevalence (55%; 66/120) than that of the gold standard microscopic (50%; 60/120) and cPCR (45.8%; 55/120) methods. The TaqMan real-time PCR method developed in this study showed high and acceptable values of these parameters suggesting that this technique could be used to increase the detect rate of *Platynosomum* spp. infection from the cats with false negative results by gold standard method.

Field of Study: Veterinary Science and technology — Student's Signature .....

Academic Year: 2020 — Advisor's Signature .....

Co-advisor's Signature .....

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Shane Thiha Soe

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

IMPORTANCE AND RATIONALE

Cats (*Felis catus*) are second most well-known companion animals in the world (Emma, 2018). Since they are carnivores, they have hunting behaviours and hunt eclectic preys ranging from rodents to insects. Lizards are one of the preys for cats in house and they can transmit the parasite that caused platynosomiasis in cats, also known as 'lizard poisoning'. *Platynosomum* spp. is the causal agent of the feline platynosomiasis and cats are acquired this parasite by ingestion of intermediate hosts such as terrestrial isopods or paratenic hosts such as house gecko and other lizards harbouring the infective stage of parasite, metacercariae (Foley, 1994).

The flukes of *Platynosomum* spp. are widely distributed in the world, especially in tropical and subtropical regions where are the common habitats of intermediate hosts with the high prevalence range of 15-81% (Basu and Charles, 2014). According to previous reports among Asian countries, the parasite has been found in Indonesia (Warren et al., 1998), Korea (Kim et al., 2010), Vietnam (Nguyen et al., 2017; Nguyen et al., 2018), Malaysia (Shanta et al., 1980) and Thailand (Jittapalapong et al., 2007).

P. fastosum is a small lanceolate cryptic hepatic parasitic species that is belong to the family of Dicrocoeliidae. The flukes commonly live inside hepatic ductal system (Maldonado, 1945) and thus, it can induce cholangitis, cholelithiasis and periductal fibrosis. In chronic and complicated case, the infection may lead to cholangiocarcinoma that can be fatal to the hosts (Andrade et al., 2012; Headley et al., 2012; Braga et al., 2016).

Generally, the *Platynosomum* infections are asymptomatic in cats but it can also lead to the progressive diseases and the host will be fatal by obstructive biliary diseases and hepatic failure (Basu and Charles, 2014). Hence, it is indicated that the diagnosis of the infections of *Platynosomum* spp. cannot be able to rely on the clinical signs and symptoms. Although there is no absolutely accurate diagnostic method of this infection, the common five coprological microscopic examinations using direct smear technique, flotation techniques with zinc sulphate, sucrose and modified detergent and also formalin-ether sedimentation techniques have been

described to detect *Platynosomum* spp. eggs in cat feces (Basu and Charles, 2014). Despite of the cheapness and simple equipment requirements of the fecal microscopic examination, it still has limitations such as considerable expertise and experience required for accurate interpretation since it is mainly based on morphological features of the eggs (Aklakur et al., 2016).

Nguyen et al. firstly attempted to identify *Platynosomum* spp. that naturally infected cats in Vietnam by using conventional PCR technique and provided the sequence data (nuclear ribosomal ITS1 region) (Nguyen et al., 2017). A recent study from Brazil had provided the information about the isolates obtained from cats of Americas and Asia were the same *Platynosomum* spp. and also proved that the isolates from different host and different geographical areas were conspecific in molecular identification (Pinto et al., 2016). Both of previous studies had utilized the known adult worms to identify as *Platynosomum* spp. by using conventional PCR techniques (cPCR). Up to the present, the information about the molecular diagnostic approach to study *Platynosomum* spp. eggs in cat feces are still scarce and limited.

Objectives

1. To develop the rapid and sensitive PCR-based methods for detection of *Platynosomum* spp. infection in cat fecal samples.
2. To compare the sensitivity and specificity of microscopic examination, conventional PCR (cPCR) and TaqMan real-time PCR methods.

Hypothesis

The PCR-based methods (conventional PCR and TaqMan real-time PCR) have higher specificity and sensitivity than the conventional microscopic examination.

Keywords (Thai): แมว ปฏิกิริยาลูกโซ่โพลิเมอเรสแบบดั้งเดิม วิธีการวินิจฉัย มูลพยาธิใบไม้ในตับแมว ปฏิกิริยาลูกโซ่โพลิเมอเรสแบบแท็คแมนเรียลไทม์
Keywords (English): Cat, Conventional PCR, Diagnostic methods, Feces, *Platynosomum* spp., TaqMan real-time PCR

CHAPTER 2
LITERATURE REVIEW

2.1 *Platynosomum* spp. in cats

The *Platynosomum* spp. flukes under the Genus *Platynosomum* termed by Looss in 1907 are belong to the family Dicrocoeliidae and they are the etiologic agents of platynosomiasis, majorly dwelling within the ductal systems of liver and gall bladder as well as inside the gall bladder. Although felids are definite hosts, the infection was also occurred in birds and other mammals, as reported in many countries such as Europe, Africa, Asia, North, Central and South America (Toledo and Fried, 2014). *P. fastosum* (=*P. illiciens*) (Pinto et al., 2016) is responsible for feline platynosomiasis, clinical signs favor to severe progressive hepatic diseases since liver fluke resides in biliary system (Rocha et al., 2014). The body of fluke is flat and elongated body, measuring of 2.8–6.8 mm long and 0.85–2.6 mm wide (Fig. 1A). The mature eggs of this parasite are usually golden-brown, thick, operculated and symmetrical features with the measurement an average of 34–50 µm x 20–35 µm (Ferreira et al., 1999) and immature eggs are mostly transparent and slightly elliptical in shape, measuring 27.2–30.0 µm × 16.3–20.0 µm (Palumbo et al., 1974; Montserin et al., 2013).

Generally, the *Platynosomum* infections are asymptomatic in cats but in some cases, the common clinical signs are showed in cats such as low body condition score, harsh respiratory sounds, ascites, hair loss (may be intermittent), anorexia, diarrhea, vomiting, emaciation, lethargy, depression, slight fever (102.4°F or 39.1°C), dehydration, dull coat and associated with hepatic diseases such as progressive icterus and jaundice (Soulsby, 1968; Bielsa and Greiner, 1985; Xavier et al., 2007; Lenis et al., 2009; Montserin et al., 2013). The intensity of clinical signs by this parasite is usually depend on three main factors; the parasite burden, the time of infection and the response of host to parasite (Salomão et al., 2005). Because of their common habitats, the parasite might cause the gallbladder wall thickening and biliary ducts dilatation, subsequently aggrandize the development of non-suppurative cholangiohepatitis and periductal fibrosis. The cholangiohepatitis is characterized by histo-pathological features with the bile duct fibrosis, the

hyperplasia of the ductal epithelium and the infiltrations of leukocytes, cholecystitis and cholangitis are also accounted (Andrade et al., 2012; Headley et al., 2012; Braga et al., 2016; Cullen and Stalker, 2016). In chronic and complicated cases, a relation of the *Platynosomum* infection and the appearance of the cholangiocarcinoma in cats had been reported (Andrade et al., 2012).

2.2 Life cycle and distribution

The life cycle of *Platynosomum* spp. is indirect as common as other trematodes and is believed to require at least two intermediate hosts of different species in order to maturate to be the adult flukes. The adult parasites commonly live in the duct systems of liver and gall bladder as well as even inside the gall bladder of felids and it can often be found in the pancreatic duct in some cases as the major duodenal papilla of pancreas is usually joint with the common bile ducts in cats (Köster et al., 2017). The parasite initiates to produce mature embryonated eggs in 12 weeks after entering the definitive hosts (Taylor and Perri, 1977). The infected cats excrete the mature eggs along with the feces and then the eggs are ingested by the first intermediate hosts, the terrestrial mollusks. After ingestion of mature eggs, the operculum of eggs opens within approximately 15 minutes inside the crop of the mollusks and ciliated miracidia are liberated altogether with other ova contents. The free-living miracidia begin to penetrate via connective tissues and migrate to the respiratory system of the mollusks. After five days of being free-living miracidia, they cease their host tissue penetration activity and lose their locomotive ability and then they transform into the mother sporocysts. The mother sporocyst contains four germ cells enclosed by a membrane. After undergoing a series of mitosis, it eventually reaches its maturity and able to produce numerous daughter sporocysts with well-developed musculature containing 18 fully developed cercariae in each within approximately 28 days. The mature daughter sporocysts make their departure from the mollusks into the environment by migration through respiratory epithelium to respiratory cavity and expulsion via the shell aperture of the mollusks. The second intermediate hosts are terrestrial coprophage isopods or beetles and they are assumed to acquire the cercariae contained mature daughter sporocysts by

ingestion. The infective stage, metacercaria, is developed in the second intermediate host and the previous published papers stated that there are two forms of metacercaria; encysted metacercaria that enclosed by a thin membrane and excysted metacercaria without membrane. The previous study revealed that the encysted metacercaria was found in the terrestrial isopods and the excysted metacercaria can be found in the amphibians and lizards. However, the amphibians and reptiles such as a lizard, gecko, frog and/or toad are not the obligatory intermediate hosts although they might serve as paratenic hosts (Arceo et al., 1999). The cats can naturally be infected by ingestion of an intermediate and/or paratenic hosts that harbouring metacercariae via predation (Foley, 1994). Nevertheless, the direct acquirement of metacercaria from terrestrial isopods to the definitive host is still skeptical and controversial. After ingestion of metacercariae, they migrate through the alimentary canal to their specific habitat sites and they are assumed to develop completely into adult parasites within eight to twelve weeks in definitive hosts (Ferreira et al., 1999). After maturation of the parasite, an individual mature worm can able to produce from 10 to 100 eggs per day (Soulsby, 1968; Palumbo et al., 1974; Basu and Charles, 2014). According to the predator-prey relationship established by mother nature, the hunting behavior of felids naturally perpetuate the life cycle of this parasite (Foley, 1994).

The prevalence of this parasite with the range of 15-81% had been reported commonly in tropical and sub-tropical countries where the environmental circumstances are favorable for the development of intermediate hosts in bountiful quantity (Bielsa and Greiner, 1985; Foley, 1994; Ferreira et al., 1999; Rodriguez-Vivas et al., 2004; Haney et al., 2006; Xavier et al., 2007; Andrade et al., 2012). Asian countries such as Indonesia (Warren et al., 1998), Korea (Kim et al., 2010), Vietnam (Nguyen et al., 2017; Nguyen et al., 2018), Malaysia (Shanta et al., 1980) and Thailand (Jittapalapong et al., 2007) been reported for *Platynosomum* spp. infection, as well as other countries such as Brazil (Mundim et al., 2004; Salomão et al., 2005; Xavier et al., 2007; Carreira et al., 2008; Andrade et al., 2012; Braga et al., 2016; Ramos et al., 2016), Mexico (Rodriguez-Vivas et al., 2004), USA (Ash, 1962; Robinson and Ehrenford, 1962; Greve and Leonard, 1966; Powell, 1970; Palumbo et al., 1974; Barriga et al.,

1981; Headley et al., 2012), Bahamas (Leam and Walker, 1963), Venezuela (Basu and Charles, 2014), Columbia (Lenis et al., 2009), Cayman Islands (Headley et al., 2012), St. Kitts (Krecek et al., 2010), Trinidad (Montserin et al., 2013), New Guinea (SAAD et al., 1984) and Nigeria (Soldan and Marques, 2011). In ASEAN countries, the occurrence of *Platynosomum* spp. had been reported with the prevalence of 9.76 – 73 % in Malaysia (Retnasabapathy and Prathap, 1971; Zain et al., 2013) and 4.91% in Vietnam (Nguyen et al., 2018). At the outset of the parasite discovery in Thailand, the prevalence was very low and then the prevalence had been documented on the increase steadily year by year from various studied provinces (Jittapalapong et al., 2007; Muksombat et al., 2008; Rojekittikhun et al., 2008; Rojekittikhun et al., 2014; Rojekittikhun et al., 2015; Jitsamai et al., 2021) (Figure 1).

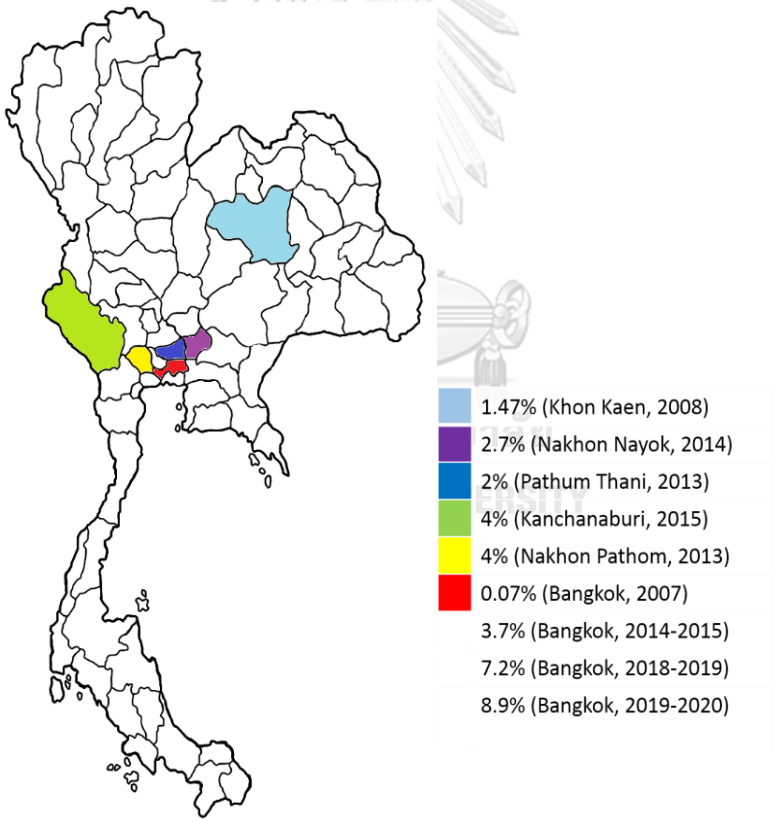


Figure 1 The prevalence of *Platynosomum* spp. reported from various provinces of Thailand

2.3 Treatment

The praziquantel utilization in combination with nitroscanate could reduce the mature egg production (Evans and Green, 1978). The study in 2018 conducted the experiment with two groups of cats by trailing two praziquantel treatment regimens; a high-dose treatment of 20 mg/kg body weight (BW) administered intramuscularly (IM) once a day for three consecutive days and a low-dose treatment of 5 mg/kg BW administered once (IM) and booster dose on 14 days later. However, this study found out that there was no satisfactory effect on absolute deprival of *Platynosomum* parasites from the infected cats when they evaluated with post-mortem fluke count (Lathroum et al., 2018).

2.4 Diagnosis methods for detection of *Platynosomum* spp. infection

As it was embarked above, the diagnosis mainly focusing on clinical signs cannot be reliable since infections are mostly asymptomatic in nature (Bielsa and Greiner, 1985). Hence, tentative diagnosis of *Platynosomum* infection in cats can be made by history taking, imaging techniques and laboratory assessment such as blood chemistry tests in conjunction with fecal microscopic examination and/or demonstration of the adult flukes in the liver/gallbladder by post-mortem (Palumbo et al., 1974; Salomão et al., 2005).

In the current scientific literatures, there is no absolute accurate test for the diagnosis of *Platynosomum* spp. (Rocha et al., 2014; Ramos et al., 2016) and at least five coprological microscopic examination techniques are used to detect the eggs in infected cat feces. They are direct smear method, flotation methods using zinc sulphate, sucrose and modified detergent floatation and also sedimentation methods with formalin ether (Basu and Charles, 2014). Among these methods, the formalin-ether concentration technique showed 100% positive results in the infected cats whereas other showed 25-50% positive (Palumbo et al., 1974). However, some papers revealed that the centrifugal floatation procedure in Sheather's sugar solution was the most effective and suitable for detection of *Platynosomum* spp. eggs in feces (Rocha et al., 2014; Eisenbraun et al., 2020).

2.5 Molecular approach for detection of *Platynosomum* spp. infections

Although the conventional microscopic examination is useful in the detection of endoparasitic infections, there are some limitations such as a heavy reliance technical experience and also sub-sampling errors (Hunt and Lello, 2012). Since the fecal egg shedding rate (eggs per gram – EPG)of *Platynosomum* spp. is relatively low (low burden with 2-10 EPG and high burden with 10-100 EPG) and also, there may be the occurrence of high morphological variation between mature and immature forms and misinterpretation of the similar morphological features of other parasites such as *Eurytrema* spp., the microscopic detection has shown to be problematic for detection of parasite eggs in cat feces (Basu and Charles, 2014). Nowadays, molecular technique has been being established in the detection of parasite in order to overcome the limitations of conventional methods.

In the past decade, rapid and accurate molecular DNA-based tests were being developed in order to overcome some limitations of the traditional methods *(Hunt and Lello, 2012)*. DNA-based techniques are applicable and being used for the diagnosis of the endoparasitic infections in both clinical and research laboratories (Ayana et al., 2019). Among the molecular techniques, PCR-based detection technique is one of the powerful tools among molecular methods since it revealed high percentage of sensitivity and specificity, depending on the genetic markers used. The diagnostic approach for endoparasitic infections by using PCR DNA-based techniques have been successful and well documented in other helminth infections such as *Opisthorchis viverrini* (Trematoda), *Echinococcus granulosus* (Cestoda), *Strongyloides stercoralis* (Nematoda) etc. (Gordon et al., 2011). On regards of genomic sequencing and easy to generate the data, the feasibility of developing PCR-based methods has been subsequently increased as the diagnostic tools for helminth infections.

In cPCR, a specific targeted sequence is amplified by a pair of primers (forward and reverse) resulting the amplicons. The amplicons of the correct size (sequence) are detected and size-discriminated by using gel electrophoresis. This technique is widely used to determine the species of parasite even in any developmental stage, since genomic DNA is easy to amplify from all type of sample

such as tissue, body fluid including feces, urine, whole blood or serum even it cannot determine for the intensity of parasite infection. In order to overcome this limitation of the cPCR technique, real-time PCR (qPCR) assays have been developed which enable to detect multiple distinct helminth species in an individual host and to determine the estimation of egg numbers in the samples (Verweij et al., 2007; ten Hove et al., 2008; Basuni et al., 2011; Taniuchi et al., 2011). During each cycle of qPCR, the fluorescence signal increases proportionally to the amount of amplified DNA molecules and thus, the targeted DNA sequence is quantified in 'real-time'. The double-stranded DNA binding dye such as SYBR green is used to allow the fluorescent labelling quantifies the amplified DNA molecules in dye-based qPCR. However, the dye-based qPCR can only examine one target at a time because the dye will bind to any possible double-stranded DNA presented in examined sample during amplification process. In probe-based qPCR, the specifically optimized and designed probes labelled with fluorophores and/or quenchers such as TaqMan probes are used in addition to primer pairs. The probe-based qPCR enables to detect many targeted sequences simultaneously in each sample and it is more specific than the dye-based qPCR. In the presence of large amount of DNA template, a rapid amplification of the intended DNA sequence region flanked by the forward and reverse primers will be occurred during the real-time PCR process. By either spiking samples or serial dilution methods, a standard curve could be generated which is able to estimate an amount of DNA in a sample. The cycle threshold value (Ct) and relative fluorescence units (RFU) was basically used to determine the approximate amount of targeted parasite egg DNA in a fecal sample (Verweij et al., 2007; ten Hove et al., 2008; Kim et al., 2009).

Among the genetic markers, ribosomal gene has approved as a useful tool to differentiate the organisms at the level of genus, species and population as it consists high conserved coding area that separated alternatively with various spacer regions. These spacers showed low intraspecific variation due to the facts of molecular drive and concerted evolution. The concerted evolution homogenizes sequences through gene conversion and unequal crossing over. The molecular drive is the one which fixes a gene variant throughout a species via random genetic drift

and natural selection (Hillis and Dixon, 1991). Two internal transcribed spacers, ITS1 and ITS2 separated the coding regions, the first is located between 18S and 5.8S and second embedded between 5.8S and 28S respectively. The selection of prospective genetic marker should have a relative exhibition of low intra-specific variation and a high level of divergence between adjoining related species and furthermore, it is fundamental that the selected genetic marker lacks intra-individual variations (Vilas et al., 2005). Due to the molecular turnover mechanisms, the ITS markers are conditionally presumed that they have low intra-specific and intra-individual variations. Moreover, the ITS markers are presumedly sharing various types among the flatworm species by being caused due to the slow concerted evolution and processes such as hybridization, incomplete lineage sorting and/or retention of ancestral polymorphism. Therefore, ITS regions of rDNAs are believed to provide profitable taxonomic information of digeneans (Hillis and Dixon, 1991) that is especially used as useful genetic markers for elucidating interspecies and intraspecies relationships among closely related genera in many eukaryotes including cryptic species such as *Dicrocoelium* spp. and *Eurytrema* spp. (Orosová et al., 2010; Yamada et al., 2012; Mohanta et al., 2015). Ergo, the usage of this marker could confound other genetic markers in molecular territory as a prospecting study (Rollinson et al., 1990; Huang et al., 2004; Vilas et al., 2005).

For the molecular approach, Nguyen et al. from Vietnam firstly attempted to identify *P. fastosum* using cPCR method and provided the sequence data (nuclear ribosomal ITS1 region) in NCBI under accession no. KU987672-KU987674 (Nguyen et al., 2017). A recent study from Brazil mentioned three isolates obtained from cats and other susceptible animal species of Americas and Asia were the same *Platynosomum* spp. and proved that the parasites from different hosts and different regions were conspecific (Pinto et al., 2016). In their study, the nuclear ribosomal ITS, 28S, and mitochondrial cytochrome c oxidase (*cox*1) sequences for this parasite attained from naturally infected cat, marmoset, lizard, and snail revealed that there were no significant molecular differences in their genetic comparisons. Both previous studies used only adult worms to identify as *Platynosomum* spp. with cPCR method.

Until now, the information about the diagnostic molecular approach to detect *Platynosomum* spp. eggs in feces is still scarce and limited.

CHAPTER 3
MATERIALS AND METHODS

3.1 Sample preparations

The sample size is derived from the sample size tables with regards to sensitivity and specificity analysis using Power Analysis and Sample Size (PASS) (Bujang and Adnan, 2016). The total number of 120 samples used for this project was retrieved from the remaining fecal sediments previously screened by microscope and recorded results that were kept in 1.5 ml centrifugal tubes at -20℃ from previous study (2019 – 2020) proceeded under IACUC no. 1931058 at the parasitology unit, 60th years Anniversary Building, Faculty of Veterinary Science, Chulalongkorn University. Based on previous conventional microscopic examination, these samples were examined prior by PBS-ethyl acetate centrifugal sedimentation technique with the results of *Platynosomum* single infection, co-infection with other parasites, other parasite infections and no detected parasites in examined samples (Figure 2). The current study was conducted under the IBC no. 2031042. The number of samples that selected to be tested by the cPCR and TaqMan real-time PCR methods in this study was equal in positive and negative samples i.e., 60 positive samples and 60 negative samples were used in the current study. Therefore, the prevalence of the infection for this study was assumed as 50% (Appendices A).

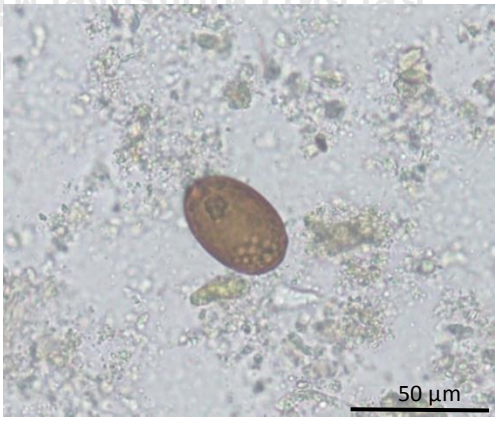


Figure 2 Eggs of *Platynosomum* spp. isolated from cat feces

3.2 DNA extraction

The adult worms retrieved from naturally infected cats kept at -80℃ that were used as positive control in this study. GeneAid Tissue Genomic DNA Mini Kit (Geneaid Biotech, New Taipei, Taiwan) was used to extract genomic DNA (gDNA) from worms. In the procedure of fecal DNA extraction, the fecal samples were repeatedly washed three times with distilled water by using a centrifuge (Phuphisut et al., 2014). The supernatant was discarded and two modified cycles of freezing the sediments rapidly in liquid nitrogen (-196 ℃) followed by incubation in a heating block set at 100℃ for 3 min (Demeler et al., 2013) in order to crack the egg shells and make the feasibility to gash the shell by bead-beating method. Later, the Qiagen TissueLyser LT bead-beating machine (Qiagen, Hilden, Germany) was used as prior mechanical lysis and then QIAamp Power Fecal Pro DNA kit (Qiagen, Hilden, Germany) was used to pursue DNA extraction of the *Playtynosomum* eggs in the feces according to the manufacturer's instructions. The derived gDNA was quantified by NanoDrop™ Spectrophotometer (ThermoFisher, Massachusetts, USA) and qualified by gel electrophoresis.

3.3 Oligonucleotide primer design

Partial ITS1-5.8S sequences of *Platynosomum* spp. under accession numbers MT015704.1, MT015703.1, MT015695.1, KU987673.1, KU987674.1 and KU987672.1 were retrieved from GenBank database (www.ncbi.nlm.nih.gov/). Sequences were aligned by using ClustalW option implanted in BioEdit Clustal Omega software. Primers and probes for cPCR and TaqMan real-time PCR methods were designed from the identical length of the retrieved sequences by using Primer3 software (https://bioinfo.ut.ee/primer3-0.4.0/). The newly designed primer sets were further tested in silico by using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm their specificity in GenBank database. The primer sets and the probe to be used in this study are shown in Table 1 below (Figure 3).

Table 1 Oligonucleotide primers and TaqMan probe that were employed in conventional PCR and TaqMan real-time PCR for detection of *Platynosomum* spp. infection

No.	Primer name	Sequence (5'-3')	bp	GC%	T_m (°C)	Self complementarity	Self 3' complementarity	Amplicon Size	Remark
1	cPF_F	TGTTCGGGGTGCCTACCTGT	20	60	68.7	3.00	2.00	350	Conventional PCR
	cPF_R	AGTGATCCACCGCTCAGAGT	20	55	65.9	5.00	3.00		
2	rtPF3_F	ATCCTGTGATATGCCTGCGG	20	55	59.96	4.00	2.00	166	Real-time PCR
	rtPF3_R	GCCCCCACGAAACTTTACCA	20	55	60.54	5.00	0.00		
3	rtPF3_Pro	FAM - TCGTGGGGACGGGGTGTACTG - BHQ	21	66.67	59.91	4.00	1.00		TaqMan probe

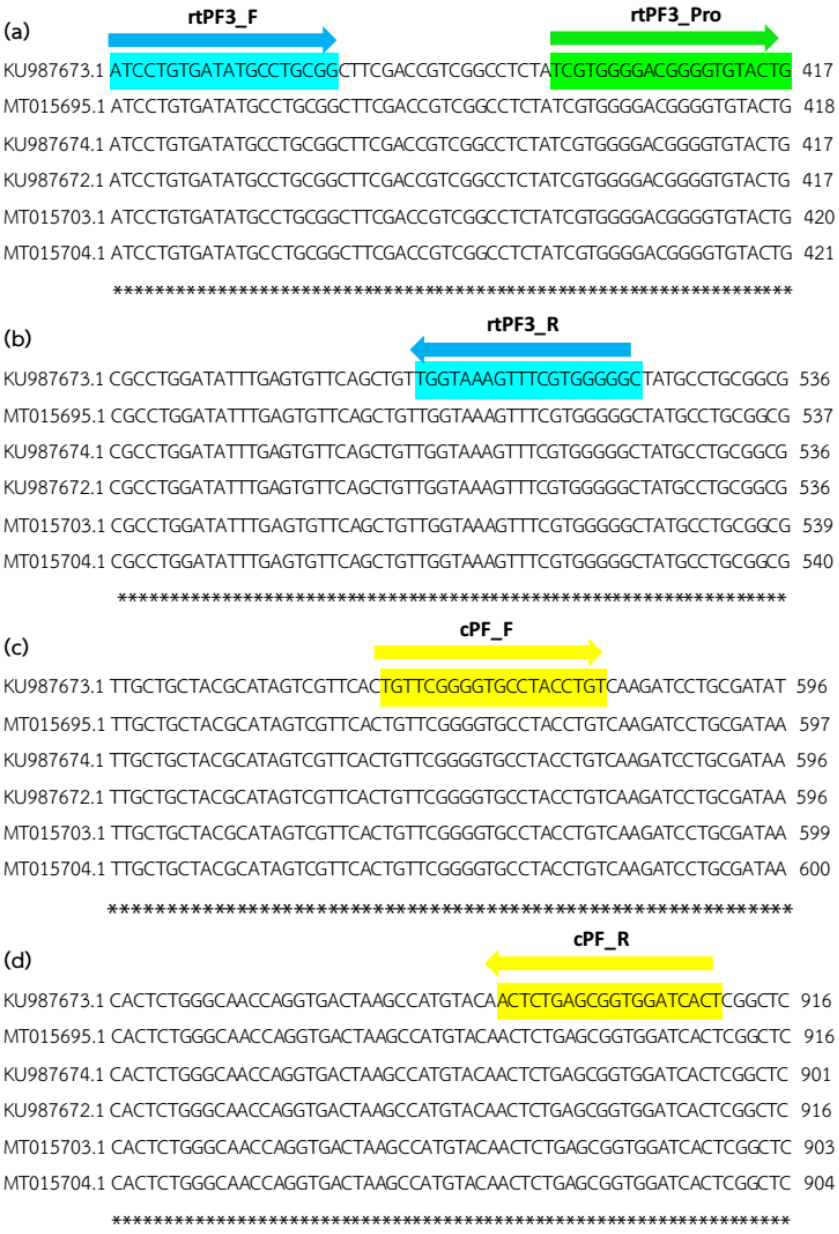


Figure 3 Specific amplification sites of forward and reverse primers and TaqMan probe sequences of conventional PCR and real-time PCR methods for *Platynosomum* spp. (a) highlighted in blue: real-time PCR forward primer sequence; highlighted in green: TaqMan probe sequence (b) highlighted in blue: real-time PCR reverse primer sequence (c) highlighted in yellow: conventional PCR forward primer sequence (c) highlighted in yellow: conventional PCR reverse primer sequence.

* means identical nucleotides.

3.4 Construction of standard plasmids

The extracted gDNA of adult worms was used as a template and amplified by newly developed primers and reaction conditions. Using positive PCR products as a template, the amplified positive PCR product was cloned into pGEM®-T easy vectors (Promega Corporation, Madison, USA) and transformed into JM 109 Escherichia coli K12 competent cells. The target DNA fragment inserted plasmids were isolated from the cultured bacterial cells by using Presto™ Mini Plasmid Kit (Geneaid Biotech, New Taipei, Taiwan). The isolated plasmid DNAs were sequenced for verification and used for determination of sensitivity rate in PCR techniques.

3.5 Analytical specificity and sensitivity of conventional PCR and TaqMan real-time PCR methods

Analytical specificity of each pair of primers was tested with the gDNAs of *Platynosomum* spp. adult worms. Cross amplification with other unintended parasites was evaluated. The common feline gastrointestinal parasites; *Ancylostoma* spp., *Toxocara* spp., *Dipylidium* spp., *Taenia taeniaeformis*, *Strongyloides* spp., *Opisthorchis viverrini*, *Spirometra* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., *Giardia* spp. and *Cystoisospora* spp. were accessed for the specificity of primer sets of each PCR assays (Appendices B).

Analytical sensitivity of the newly developed protocols was performed to detect the target DNA fragment by making a serial dilutions of known copy number of recombinant plasmids. DNA concentration of cloning plasmids were measured by using NanoDrop™ Spectrophotometer (ThermoFisher, Massachusetts, USA) and plasmid copy number were determined by using DNA copy number and dilution calculator software provided by ThermoFisher (https://www.thermofisher.com). The protocol of sensitivity was conducted by a serial 10-fold dilutions ranging from 10^1-10^6 copy numbers per microliter. The limit of detection (LoD) was assessed for the primer sets of both cPCR and TaqMan real-time PCR methods. Standard curve of known copy number of recombinant plasmids as well as known DNA concentration of adult worm gDNA spiked into negative fecal DNA samples (100 ng/µl) that was modified and adopted from the procedure of previous study (Worasith et al., 2015)

relative to positive quantification cycle (Cq) scores was determined for TaqMan real-time PCR method.

3.6 Validation of conventional PCR and TaqMan real-time PCR methods in cat fecal samples

The cPCR and TaqMan real-time PCR methods were performed by using modified methods that were described in the previous published papers (Nguyen et al., 2017; Adisakwattana et al., 2020). Before amplification procedure, the DNAs of field samples were diluted in the ratio of one-fifth with Mili-Q water to avoid the inhibitory effects of the PCR inhibitors presented in fecal samples (Cao et al., 2012; Acharya et al., 2017) and then the amplification of cPCR was optimized and performed in a 20 µl final volume containing; 10 pmol of each primer, approximately 10 ng/µl of DNA, 1U of 1 x DreamTaq DNA polymerase (ThermoFisher, Massachusetts, USA), 2 µl of Dream Taq buffer, 0.2 mM of dNTP and Mili-Q water. The amplification of the partial sequence of ITS1-5.8S region was done with T100™ Thermal Cycler (Bio-Rad, California, USA) using the following cycling conditions; 3 min initial denaturation step at 95˚C, 35 cycles of 30 sec at 95˚C, 30 sec at 58˚C and 20 sec at 72˚C, and a 5-min extension at 72˚C. To ensure that no PCR inhibitors were present in the PCR reaction, the PCR amplification that yielded a negative result was then repeated by spiking with the positive DNA.

The TaqMan real-time PCR was performed in a final reaction volume of 20 µl in which 2 pmol of each primer, 5 pmol of TaqMan labelled probe, 10 µl of 2 x iTaq universal probes supermix (Bio-Rad, California, USA), approximately 10 ng/ µl of DNA and Mili-Q water. The amplification was performed in a Bio-Rad CFX96 Touch real-time PCR machine (Bio-Rad, California, USA) with the optimized thermal cycle conditions; 3 min initial denaturation step at 95˚C followed by 40 cycles of 10 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C. The gDNAs of adult worms and eggs and target DNA fragment inserted plasmids were amplified by the modified and optimized PCR conditions before it was applied to field samples.

3.7 Data analysis

In this study, the previous results screened by the microscope used as 'gold standard'. Sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), likelihood of observing a positive test result in infected animal (LR+) and the likelihood of observing a negative test result in non-infected animal (LR-) (after subtracting from 1) were calculated comparing with gold standard method. LRs are fundamental ratio of the probability that a test result is correct to the probability that the test result is incorrect. The magnitude of probability is determined according to the LR+ and LR- absolute values that described by Edman and Runge (Edman and Runge, 2014). The magnitude of probability is ranked into minimal probability (LR+: 1 - 2 and LR-: 0.5 – 1), moderate probability (LR+: 5 - 10 and LR-: 0.2 – 0.5) and high probability (LR+: >10 and LR-: 0.0 – 0.2). Using MedCal (MedCalc Software, Ostend, Belgium), the diagnostic accuracy of the newly developed PCR techniques for *Platynosomum* spp. was estimated in terms of sensitivity, specificity, PPV and NPV based on the predetermined prevalence value of 50%. The PPV and NPV values are variable according to the prevalence value of infection.

The McNemar's chi-squared test was used to compare the result to determine the significant difference between individual PCR methods and gold standard of the present study. Since the McNemar test is usually done on in a 2 x 2 table and thus, the degree of freedom for this statistic is 1. The critical value for the current test is 3.84 that derived from the chi-square table for the test with $\alpha = 0.05$ (95% CI). The null hypothesis is rejected if the McNemar value is > 3.84 and the null hypothesis is not rejected if the McNemar value is < 3.84 in vice versa ($H_0 = 0$, $H_A \neq 0$).

The test strength agreement was calculated using Cohen's Kappa statistic test along with the standard error (SE) and confidence interval (CI). Statistical analysis was performed by using R program software version 4.1.0 and EpiTools (https://epitools.ausvet.com.au/) to compare the detection rate of the microscopy,

cPCR and TaqMan real-time PCR techniques. Cohen Kappa statistic test was used to confirm the strength of agreement between two methods. In which, κ equal to zero means that the agreement was observed by chance, κ = 1 means the results from two methods were perfectly agree each other (Cohen, 1960), and then κ value ranging from 0 to 1 represents different degrees of agreement (McHugh, 2012). In this study, the classification suggested by Altman, Landis and Koch was used to interpret the strength of the agreement based on the Cohen's Kappa value (Landis and Koch, 1977; Altman, 1990). The strength of agreement was classified into poor (<0), slight (0.01 – 0.2), fair (0.21 – 0.4), moderate (0.41 – 0.6), substantial (0.61 – 0.8) and almost perfect (0.81 – 1) respectively. The formula for statistical analysis calculation is as follows (Table 2).

Table 2 Example of a sample data set used in statistical calculations.

		Test 1		Total
		Positive	Negative	
Test 2	Positive	a	b	a + b
	Negative	c	d	c + d
Total		a + c	b + d	N

McNemar's chi-square (χ^2) = $\frac{(|b-c|-1)^2}{(b+c)}$ -

Observed agreement percentage (P_o) = $\frac{(a+d)}{N}$ x 100

Expected (chance) agreement (P_e) = $\frac{\frac{(a+b)\times(a+c)}{N}+\frac{(c+d)\times(b+d)}{N}}{N}$

Cohen Kappa (κ) = $\frac{P_o - P_e}{(1-P_e)}$

Standard of error of Kappa (SE_k) = $\sqrt{\frac{P_o(1-P_o)}{n(1-P_e)^2}}$

$$\text{Sensitivity} = \frac{a}{(a+c)} \times 100$$

$$\text{Specificity} = \frac{d}{b+d} \times 100$$

$$\text{Positive predictive value (PPV)} = \frac{a}{a+b} \times 100$$

$$\text{Negative predictive value (NPV)} = \frac{d}{c+d} \times 100$$

$$\text{LR+} = \frac{\text{sensitivity}}{(1-\text{specificity})}$$

$$\text{LR-} = \frac{(1-\text{sensitivity})}{\text{specificity}}$$

Whereas,

a = True positive

b = False positive

c = False negative

d = True negative

N = Total number of samples

LR+ = likelihood of observing a positive test result in infected animal

LR- = likelihood of observing a negative test result in non-infected animal

CHAPTER 4
RESULTS

4.1 Primers and sequences analyses

In silico, the newly developed cPCR primers have a GC content 60% in forward primer and 55% in reverse primer while TaqMan real-time PCR primer pairs have a GC content 55% in each primer and 66.67% in the TaqMan probe, according to primer-BLAST data. The cPCR primers, TaqMan real-time PCR primers and probes have relatively low self-complementarity, ranging from 3 to 5 and self-3' complementarity ranging from 0 to 3, respectively. The results from Primer-BLAST of cPCR and TaqMan real-time PCR primer pairs and probe revealed that *P. fastosum* and *P. illiciens* are feasible targets to bind, but other parasites not usually detected in cats showed mismatches.

In this study, the partial sequence of ITS1-5.8S region was amplified using newly developed primer and reaction conditions. The results of PCR amplification showed a single amplicon of 350 bp for *Platynosomum* adult worms (Figure 4A) and eggs (Figure 4B). There was no amplification in the negative control (Lane 5 of Figure 4A and Lane 4 of Figure 4B).

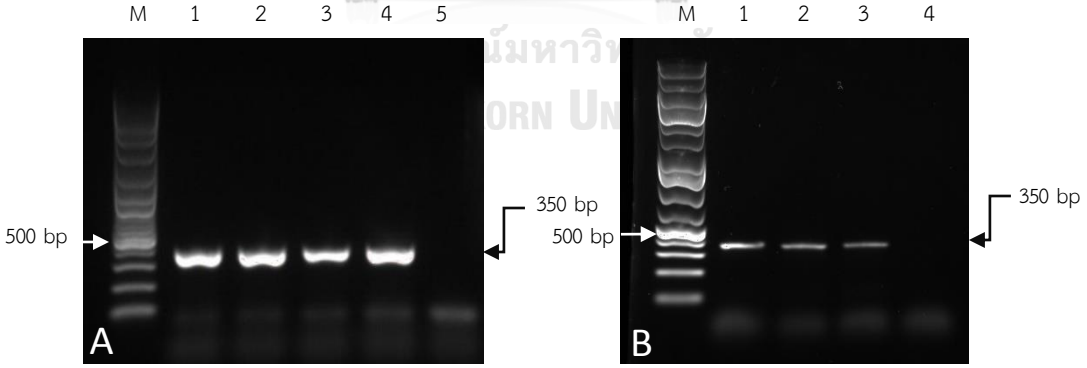


Figure 4 PCR amplicons of ITS1 region of adult worms (A) and eggs (B) of *Platynosomum* spp. were amplified using the newly developed primer and PCR condition. Lane M = VC 1kb DNA ladder (Vivantis Technologies, Malaysia), lanes 1-4 = PCR products of adult worm gDNAs and land 5 = negative control (A) Lane M = VC 1kb DNA ladder (Vivantis Technologies, Malaysia), lanes 2-3 = PCR products of egg gDNAs; and lane 4 = negative control (B).

PCR amplicons were purified and sequenced. The result found that DNA sequences from *Platynosomum* adult worms and eggs were found to have 97.07% to 100% identity with ITS1 region of *Platynosomum* spp. in the GenBank database (Table 4).

Table 3 Sequence confirmation of ITS1 partial sequences of *Platynosomum* spp. in the current study

Sample	Species	Identity %	Accession No.
P01	*P. illiciens*	100	MT015705.1, MT015700.1, MT015699.1, MT015698.1, MT015696.1, MT015694.1, MH156567.1, MH156566.1, MH156565.1, MH156564.1
		99.61	MT015697.1,
		97.99	MT015701.1
	P. fastosum	100	MT015703.1, MK166042.1, KU987674.1, KU987672.1
		99.61	MT015704.1,
		99.22	KU987673.1
P08	*P. illiciens*	99.24	MT015705.1, MT015700.1, MT015699.1, MT015698.1, MT015696.1, MT015694.1, MH156567.1, MH156566.1, MH156565.1, MH156564.1
		98.86	MT015697.1
		97.07	MT015701.1
	P. fastosum	99.24	MT015703.1, MK166042.1, KU987674.1, KU987672.1,
		98.86	MT015704.1
		98.48	KU987673.1
P23	*P. illiciens*	99.02	MT015705.1, MT015700.1, MT015699.1, MT015698.1, MT015696.1, MT015694.1, MH156567.1, MH156566.1, MH156565.1, MH156564.1
		98.53	MT015697.1,
		97.55	MT015701.1
	P. fastosum	99.02	MT015703.1, MK166042.1, KU987674.1, KU987672.1
		98.53	MT015704.1, KU987673.1

4.2 Analytical specificity of cPCR and TaqMan real-time PCR

The genomic DNAs derived from common feline parasites; *Ancylostoma* spp., *Toxocara* spp., *Dipylidium* spp., *Taenia taeniaeformis*, *Strongyloides* spp., *Opisthorchis viverrini*, *Spirometra* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., *Giardia* spp. and *Cystoisospora* spp. were amplified using newly developed cPCR and qPCR assays. There was no amplification occurred in the tested feline common gastrointestinal parasites, however, the *Platynosomum* spp. DNA was positive (Figure 5 and 6).

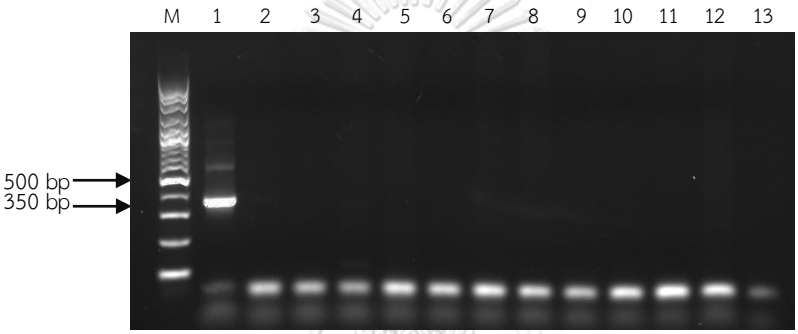


Figure 5 The gel electrophoresis of specificity investigation of cPCR method for *Platynosomum* spp. detection. Lane M: VC 1kb DNA ladder (Vivantis, Technologies, Malaysia); Lane 1: positive with *Platynosomum* species (350 bp); Lane 2: *Ancylostoma* spp.; Lane 3: *Toxocara* spp.; Lane 4: *Dipylidium* spp.; Lane 5: *Taenia taeniaeformis*; Lane 4: *Strongyloides* spp.; Lane 7: *Opisthorchis viverrini*; Lane 8: *Spirometra* spp.; Lane 9: *Toxoplasma gondii*; Lane 10: *Cryptosporidium* spp.; Lane 11: *Giardia* spp.; Lane 12: *Cystoisospora* spp.; Lane 13: negative control

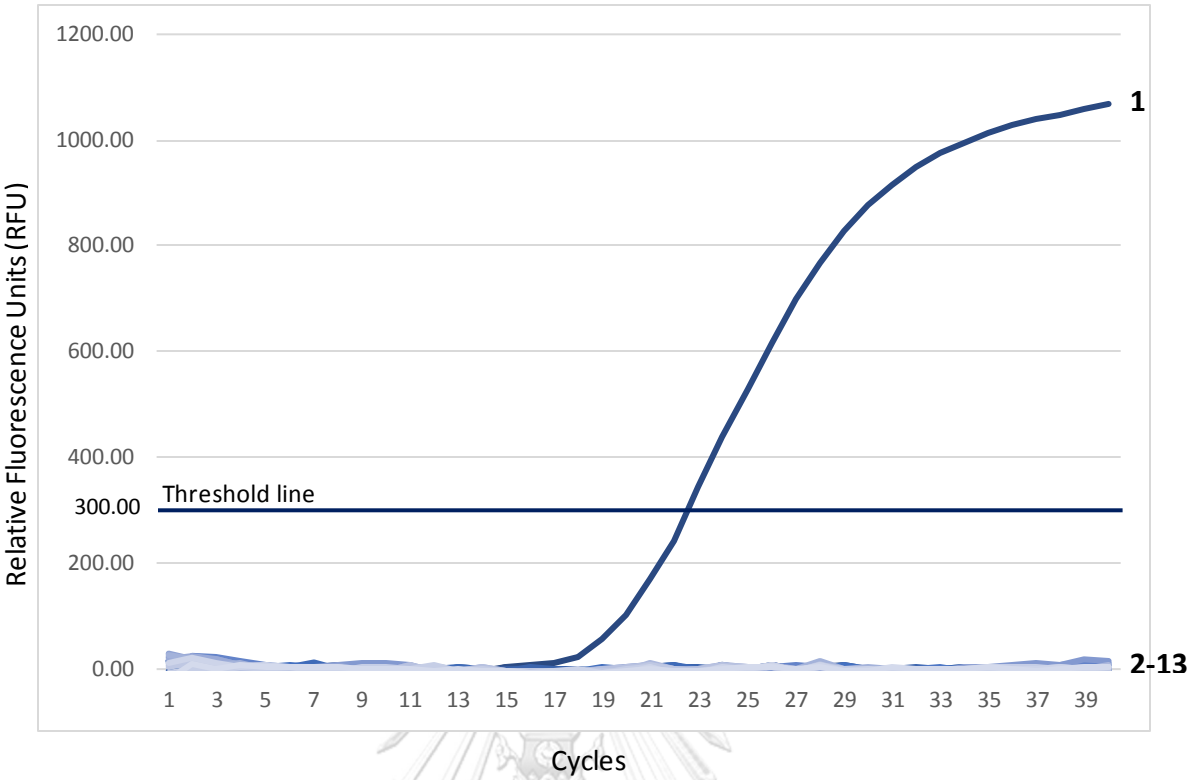


Figure 6 The TaqMan real-time PCR amplification plot for specificity analysis of *Platynosomum* spp. detection. 1: *Platynosomum* spp.; 2 - 13: Ancylostoma spp., *Toxocara* spp., *Dipylidium* spp., *Taenia taeniaeformis*, Strongyloides spp., *Opisthorchis viverrini*, *Spirometra* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., *Giardia* spp., *Cystoisospora* spp. and negative control

4.3 Analytical sensitivity (Limit of Detection, LoD) of cPCR and TaqMan real-time PCR methods

Sensitivity of cPCR was determined using ten-fold serial dilutions of plasmid containing target genes ranging from 10^6 – 10^1 copies/µl. At the concentration of 10 pmol/µl of primer in cPCR method, the newly developed primer and reaction conditions of the current study were able to detect targeted DNA template at the level as low as 10 copies/µl and as little as 0.1 ng/µl in negative fecal DNA sample (Figure 7 and 8).

The TaqMan real-time PCR method can detect the *Platynosomum* spp. gDNA and standard plasmids at the concentration as low as 0.01 ng/µl and 10 copies/µl

respectively (Figure 9 and 10). The standard curve of TaqMan real-time PCR was constructed according to the gradient results of the sensitivity test and the Cq score to define positive and negative was generated by TaqMan real-time PCR. When the relative fluorescence value (RFU) was greater than 300 RFU at 35 cycles, a sample was declared positive; when the RFU value was less than 300 RFU at 35 cycles, a sample was judged negative.

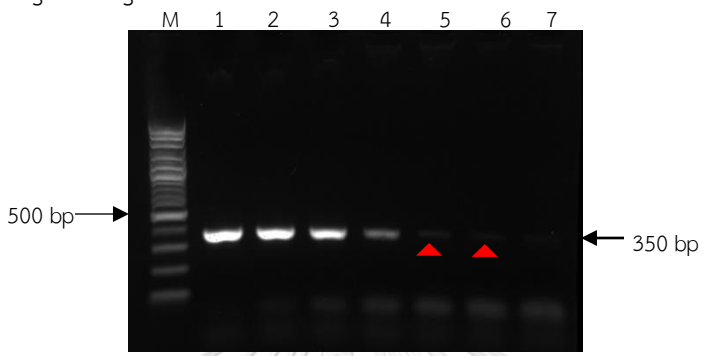


Figure 7 The gel electrophoresis of limit of detection (LoD) analysis of conventional PCR method (red arrowhead) using plasmid containing target gene as a template for detection of *Platynosomum* spp. DNA template was diluted from 10^6 – 10^1 copies/µl. Lane M: VC 1kb DNA ladder (Vivantis, Technologies, Malaysia); Lane 1: 10^6 copies/µl; Lane 2: 10^5 copies/µl; Lane 3: 10^4 copies/µl; Lane 4: 10^3 copies/µl; Lane 5: 10^2 copies/µl; Lane 6: 10^1 copies/µl; Lane 7: negative control.

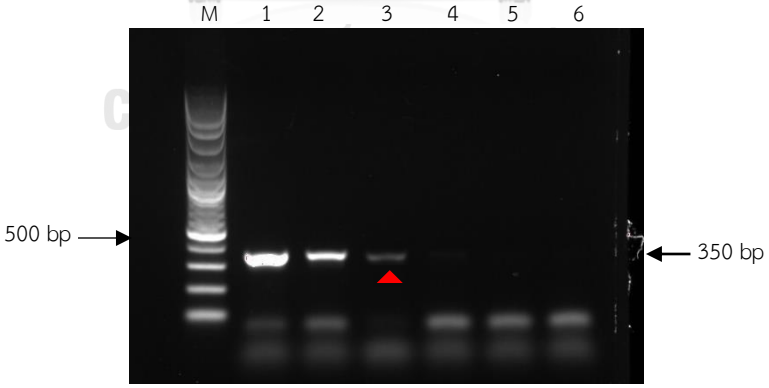


Figure 8 The gel electrophoresis of limit of detection (LoD) analysis of conventional PCR method (red arrowhead) using *Platynosomum* spp. DNA template, as a template with serial dilution (10^1 – 10^{-3} ng/µl and NC = Negative control), spiked in known DNA concentration negative fecal samples for detection of *Platynosomum* spp.

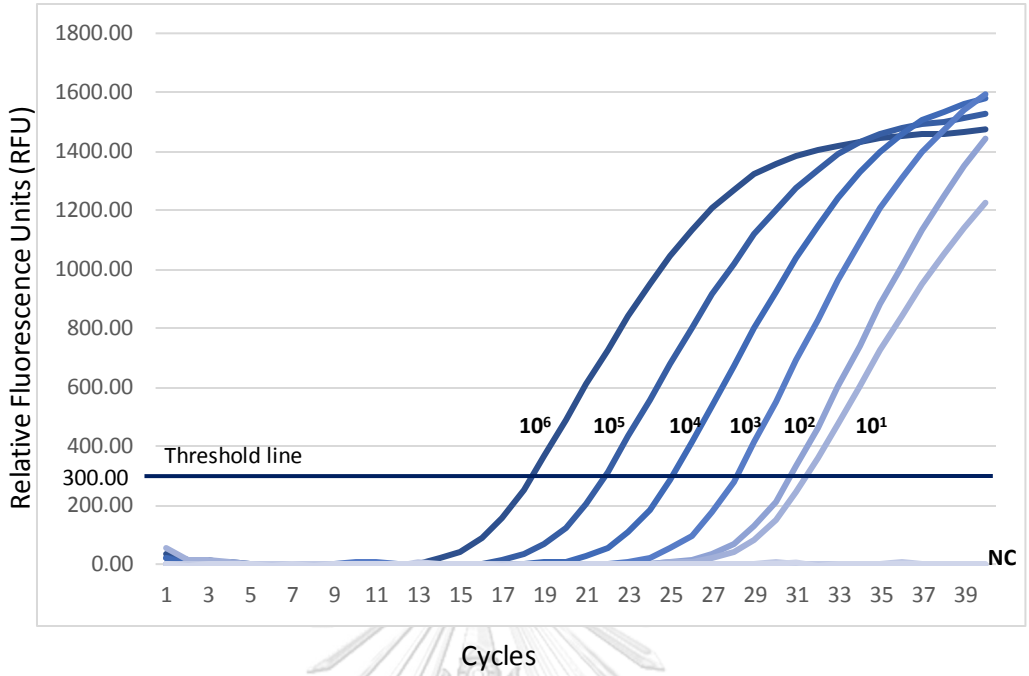


Figure 9 The TaqMan real-time amplification plot of limit of detection (LoD) analysis of TaqMan real-time PCR method using plasmid containing target gene as a template for detection of *Platynosomum* spp. DNA template was diluted from 10^6 – 10^1 copies/µl. The curve showed the amplification of plasmid containing target gene (10^6 – 10^1 copies/µl and NC = Negative control).

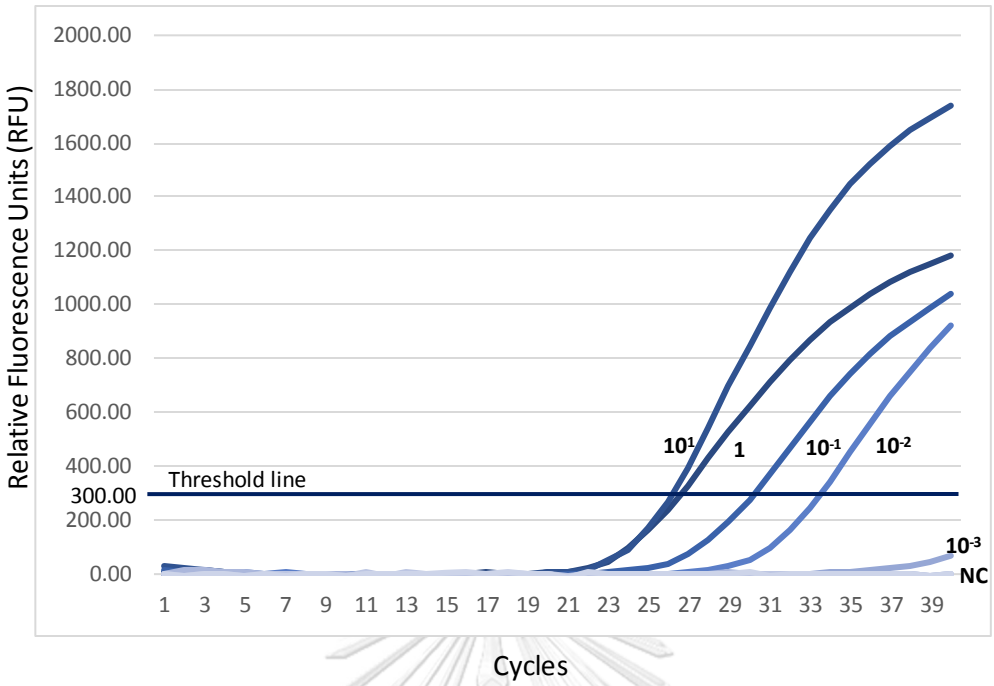


Figure 10 The TaqMan real-time amplification plot of limit of detection (LoD) of TaqMan real-time PCR method using *Platynosomum* spp. DNA, as a template with serial dilution (10^{1} – 10^{-3} ng/µl and NC = Negative control), spiked in known DNA concentration negative fecal samples for detection of *Platynosomum* spp.

4.4 Application of PCR methods to the field samples

The total number of 120 fecal sediment samples were extracted DNAs and were amplified by using newly developed primer and primer condition. These samples were priorly examined by microscope and the finding results were recorded. According to the previous results, the samples were categorized into two major groups; *Platynosomum* positive samples and *Platynosomum* negative samples. Addition to the major groups, the samples were also subdivided into two groups respectively; single *Platynosomum* infection and co-infected with other parasites under the major group of *Platynosomum* positive samples and other parasite infections and no detected parasites under the major group of *Platynosomum* negative samples (Appendices A).

The cPCR method identified a total of 55 out of 120 samples (45.83%) (Figure 11 and 13), while the TaqMan real-time PCR method revealed 66 positive results in overall samples (55%) (Figure 12 and 14) (Table 4). Interestingly, out of 60 microscopically negative samples, both cPCR and TaqMan real-time PCR techniques found 7 positive results.

Table 4 The detection rate *Platynosomum* eggs in cat feces by microscopy, cPCR and TaqMan real-time PCR methods used in this study

	Results
Test	**% (no. of positive samples/ no. of tested samples)**
Microscopy	50 (60/120)
cPCR	45.83 (55/120)
TaqMan real-time PCR	55 (66/120)

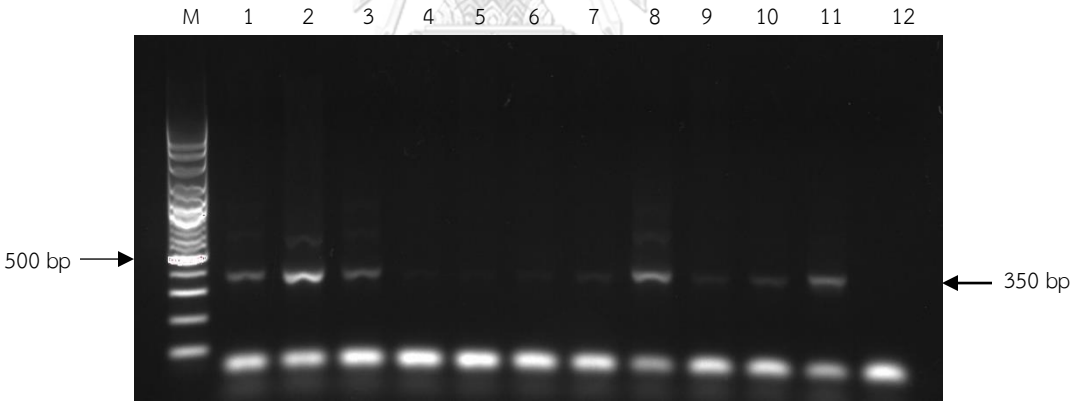


Figure 11 The gel electrophoresis of representative target amplicons that amplified from the cat fecal samples using cPCR method. Lane M: VC 1kb DNA ladder (Vivantis, Technologies, Malaysia); Lane 1 to 10: cat fecal samples; Lane 11: positive control; Lane 12: negative control.

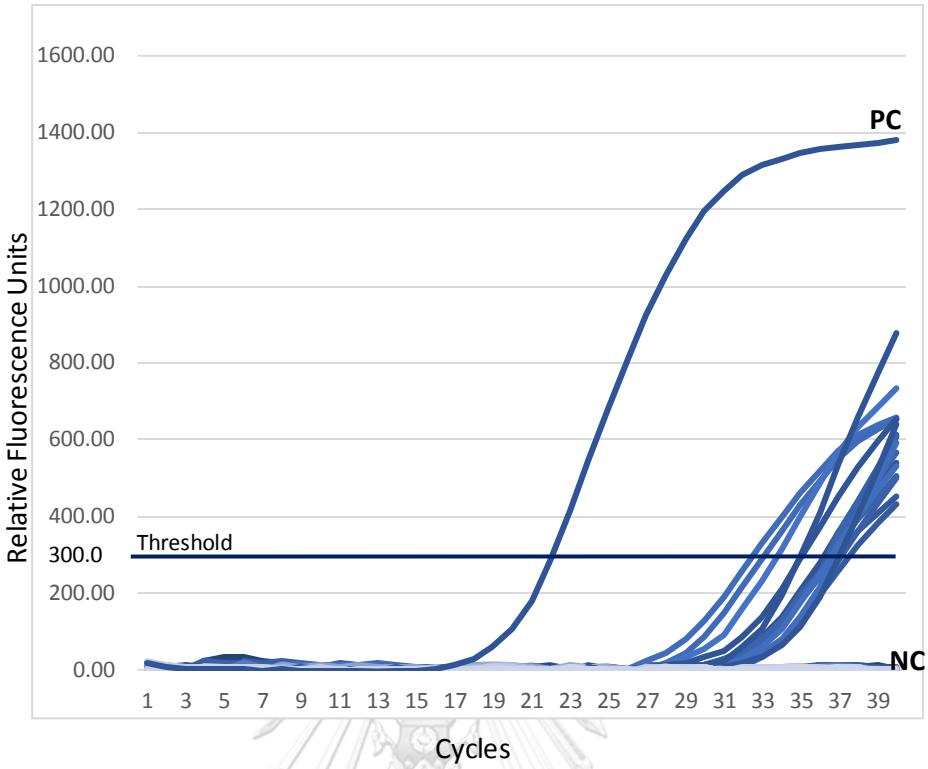
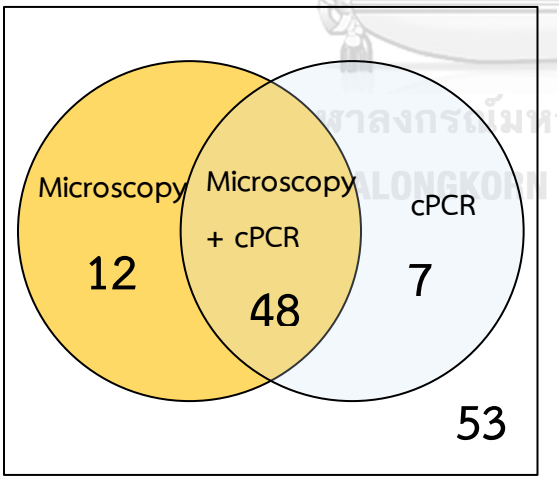


Figure 12 An amplification plot of representative cat fecal samples using TaqMan real-time PCR method. PC: Positive control; NC: Negative control

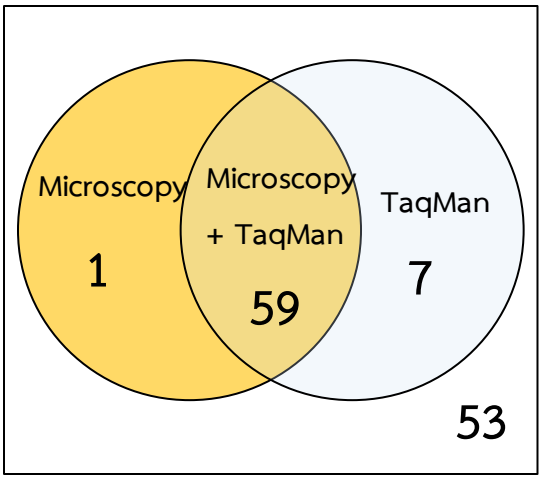


Total positive microscopy = 60 (50%)

Total positive cPCR = 55 (45.8%)

Total number of samples = 120

Figure 13 Venn diagram demonstrating the overall prevalence and agreement between microscopy and cPCR diagnostic methods used in this study.



Total positive microscopy = 60 (50%)

Total positive TaqMan = 66 (55%)

Total number of samples = 120

Figure 14 Venn diagram demonstrating the overall prevalence and agreement between microscopy and TaqMan real-time PCR diagnostic methods used in this study.

4.5 Statistical analysis of results

The raw agreement percentage (P_o) between cPCR and gold standard was 84% (True positive = 48 and True negative = 53). The McNemar chi-square value for the comparison of microscopic examination and cPCR is 0.84 and the p-value is 0.36 with 95% CI. The null hypothesis is not rejected and there is no significant difference between the two compared tests because p-value is less than 3.84. The Cohen's Kappa value of 0.68, which rounds to confidence interval of 0.55 to 0.81, indicates moderate agreement between the two comparable tests. The percentage of data reliability ranges from 35 to 63% (McHugh, 2012). When compared to the gold standard, the cPCR has 80% sensitivity and 88.33% specificity. The PPV, NPV, LR+ and LR- values are 87.27%, 81.54%, 6.86 and 0.23, respectively. The accuracy of cPCR is 84.17% (Table 5 and 7) (Figure 13).

Table 5 The contingency comparison table of microscopy and cPCR in overall samples

		Microscopy		Total
		Positive	Negative	
cPCR	Positive	48	7	55
	Negative	12	53	65
	Total	60	60	120

The agreement percentage of the observed positive samples (P_o) between gold standard and TaqMan real-time PCR method was 93% (True positive = 59 and True negative = 53). When the results of TaqMan real-time PCR method applied on field samples were compared to the gold standard, it was discovered that there is no significant statistical difference between the two tests as the McNemar's chi-square value is 3.13 and the p-value is less than 3.84 (p = 0.08). The Cohen's Kappa value of 0.87 indicates that there is a strong agreement between these diagnostic tests. The percentage of data reliability is 64 - 81% with the confidence interval of 0.78 to 0.96 (McHugh, 2012). The sensitivity, specificity, PPV, NPV, LR+, LR- and accuracy values of TaqMan real-time PCR technique are 98.33%, 88.33%, 89.39%, 98.15%, 8.43, 0.02 and 93.33% respectively (Table 6 and 7) (Figure 14).

Table 6 The contingency comparison table of microscopy and TaqMan real-time PCR in overall samples

		Microscopy		Total
		Positive	Negative	
TaqMan real-time PCR	Positive	59	7	55
	Negative	1	53	65
	Total	60	60	120

Table 7 Diagnostic performance of the *Platynosomum* spp. eggs detection by cPCR and TaqMan real-time methods was compared to the gold standard microscopic examination in field-collected samples (n = 120) based on the specified prevalence value of 50%

Comparator	Diagnostic performance parameter						
	Sensitivity	Specificity	PPV*	NPV*	Accuracy*	LR+	LR-
cPCR	80.00%	88.33%	87.27%	81.54%	84.17%	6.86	0.23
qPCR	98.33%	88.33%	89.39%	98.15%	93.33%	8.43	0.02

(*) These values are dependent on the prevalence of infection

LR+ refers to the likelihood of observing a positive test result in infected animal

LR- refers to the likelihood, after subtracting from 1, of observing a negative test result in non-infected animal

CHAPTER 5
DISCUSSIONS

Although *Platynosomum* spp. in various hosts had been reported in several countries, the intraspecific differentiation between the parasites such as *P. fastosum*, *P. illiciens* and *P. concinum* is still be controversial and they are treated to be synonymous in literature. Moreover, *Platynosomum* spp. was assumed to be synonymous with other parasites including *Concinnum concinnum*, *Dicrocelium lanceolatum var symmetricum*, *P. planicipitus*, and *D. concinnum* (Maldonado, 1945; Bowman et al., 2002; Basu and Charles, 2014). Based on the present taxonomy knowledge of the *Platynosomum* spp. and the strict information about the proper morphological comparison between *P. fastosum* and *P. illiciens*, these two putative parasites can barely be distinguished by using criteria regarding the class of the definitive host and the morphological features of testis (Pinto et al., 2016; Nguyen et al., 2017). In fact, the testis of *P. fastosum* is entire and/or slightly lobed but the testis is deeply lobed in *P. illiciens* (Lenis et al., 2009). However, the morphological polymorphism of digeneans such as cryptic species of Dicrocoeliid family is affected by several factors including parasite age, technical variations to fix and mount the parasite for morphometric measuring and the phenotypic plasticity according to the host species (Sitko, 1998). It indicated that the morphological features and their measurements are not reliable source to distinguish *P. fastosum* and *P. illiciens*. Moreover, the specificity of *Platynosomum* spp. to hosts is not the appropriate taxonomic criterion since the host specificity of dicrocoeliid parasites is low and the study of Brazil (Pinto et al., 2018a) had also revealed it by conducting the experimental infections of same isolates of *Platynosomum* spp. to birds and mammals. The authors also claimed that the name *P. illiciens* should be used as the rightful branding the etiologic trematode of feline platynosomiasis in the Americas and Southeast Asia (Pinto et al., 2018a).

The previous published papers mentioned that the re-evaluation of the diversity of species that belong to the genus *Platynosomum* should be conducted by molecular technology because they have a high intraspecific morphological variability and a high level of morphological polymorphism likely resulting from numerous

factors affecting on the parasite such as genetic variation and phenotypic plasticity (Pinto et al., 2016). Molecular approach to study *Platynosomum* spp. might provide the better understanding of this parasite relating to taxonomical intelligence as well as the evolutional theory of the parasite. Since the eukaryotic rDNA is tandemly organized high copy numbers encoding several repeat units in which including genes coding for small and large subunits and 5.8S rDNAs separated each other by spacer regions, it has been commonly used in phylogenetic approaches and diagnostic biomarker in molecular technology (Hwang and Kim, 1999).

The partial region of ITS1 was selected to use as a biomarker in this study because the ITS regions are vastly applied for identification of cryptic species such as digeneans as well as the use of ITS regions as molecular biomarkers in the applicable categorical level of genus, species and population can be less problematic than the use of small and large subunits and 5.8S rDNA (Hwang and Kim, 1999). Nguyen et al. and Pinto et al. utilized ITS1 partial region of nuclear ribosomal DNA (rDNA) to identify *Platynosomum* spp. that are found in naturally infected cats, marmosets, lizards, snails and birds from Vietnam and Brazil. Their studies showed that there are no significant molecular differences between the isolates of various hosts and the ITS1 sequences of *P. illiciens* from Brazil isolates showed high similarity with the ITS1 sequences of *P. fastosum* from Vietnam isolates. Hence, it demonstrated that the ITS1 partial sequences can be conspecific and also be used to identify *Platynosomum* spp. from different hosts and geographical regions (Pinto et al., 2018b). The combinative information presented by the studies of Brazil and Vietnam had proven that the ITS1 partial sequences of *Platynosomum* spp. can be used to detect the parasite in molecular way.

The primer set that published by Nguyen et al. was firstly used to amplify gDNAs of adult worm and eggs in the current study. There was no amplification occurred in both adult worm and eggs gDNAs. The BLAST results of this primer set showed that it was not matched with the sequences of *Platynosomum* spp. in GenBank database. The amplicon size of this primer set is 990 bp and it is quite long for diagnostic purpose. Hence, the new primer pair for cPCR was newly designed in the present study.

In order to create primers and probe for conventional PCR and TaqMan real-time PCR, the current work used ITS1 partial sequences tagged with *Platynosomum* spp. that were stored in GenBank. The gDNAs of *Platynosomum* spp. eggs and adult worm were successfully amplified with the newly developed primers and reaction conditions by showing the target amplicon size of 350 bp in electrophoresis screening in cPCR and a strong positive amplification curve in TaqMan real-time PCR. The sequencing results of the PCR products showed a high percentage of identity ranging from 97.07 to 100 with the ITS1 partial sequences of *Platynosomum* spp. in GenBank. As a result, the sequence-BLAST results showed that the cPCR and TaqMan real-time PCR techniques established in this study are capable of identifying the *Platynosomum* spp. obtained from both eggs and adult worms.

The common coprological microscopic examination methods including direct smear method, flotation methods using zinc sulphate, sucrose and modified detergent floatation and sedimentation techniques with formalin ether often used to detect the eggs in feline feces. Among these methods, the formalin ether sedimentation technique was the most sensitive technique for *Platynosomum* spp. diagnosis by showing 100% positive in all infected samples (Palumbo et al., 1974; Basu and Charles, 2014). However, some publications revealed that the centrifugal floatation procedure in Sheather's sugar solution was the most effective and suitable for detection of *Platynosomum* spp. eggs in feces (Rocha et al., 2014; Eisenbraun et al., 2020). Notwithstanding a controversial argument in fecal eggs concentration methods of *Platynosomum* spp. diagnosis, the centrifugal PBS-ethyl acetate sedimentation procedure was used to concentrate the fecal sample in this study since *Platynosomum* spp. eggs are operculated and it is needed to avoid the prior breakage of the eggshells and liberation of egg contents caused due to osmotic pressure during storage.

Nematode eggs and trematode eggs show analogous tenacity, the combined egg lysis method with freeze-boil cycles and bead-beating technique might be more or less susceptible for *Platynosomum* spp. eggs while this lysis method was functioning properly for *Trichuris* spp. eggs, strongyle and ascarid eggs (Demeler et al., 2013). It was reported that the bead-beating stage for mechanical lysis of eggs before DNA

extraction is very important and has an impact on the sensitivity and DNA recovery from fecal samples (Ayana et al., 2019). Therefore, this combination approach was used to lyse the *Platynosomum* eggs in the current study and the result showed high yielded of DNA concentration.

Naturally, fecal samples contain various PCR inhibitors such as organic and inorganic compounds, bacterial proteases, nucleases and cell debris abundantly (Repetto et al., 2013; Ayana et al., 2019). The inhibitory ability of the PCR inhibitors in fecal DNA extract was abstained by a simple one-fifth dilution with Mili-Q water in this study prior to PCR amplification. This approaching way had been utilized in animal health diagnostic laboratories in Australia (Acharya et al., 2017) and it has been agreeable with findings of previous studies by increasing the sensitivity results of 78 to 100% in environmental inhibitor-enriched water and 55 to 80% in fecal samples (Cao et al., 2012; Acharya et al., 2017).

The newly developed cPCR and TaqMan real-time PCR methods were tested for cross amplification with gDNAs from other common feline gastrointestinal parasites including; *Ancylostoma* spp., *Toxocara* spp., *Dipylidium* spp., *Taenia taeniaeformis*, *Strongyloides* spp., *Opisthorchis viverrini*, *Spirometra* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., *Giardia* spp. and *Cystoisospora* spp. The result showed that there was no evidence of cross amplification, indicating that the newly developed primer pair can specifically amplify the target ITS1 fragment of *Platynosomum* spp. in cats.

The sensitivity of in cPCR and TaqMan real-time PCR methods were determined using a standard solution of recombinant plasmids containing 10^1-10^6 copy numbers. The result showed that both cPCR and TaqMan real-time PCR methods could detect as little as ten copies number of *Platynosomum* spp. target DNA, indicating the high potential detection. Furthermore, in this study, the amount of DNA isolated from one egg of *Platynosomum* spp. was approximately 0.02 ng/µl. According to the results, cPCR and TaqMan real-time PCR methods can detect *Platynosomum* spp. DNA that spiked in 100 ng/µl of negative fecal sample DNA concentrations as low as 0.1 and 0.01 ng/µl, respectively. This suggests that the PCR methods described here can

detect the *Platynosomum* eggs in cat feces even if only one egg is present in the sample.

Because this is the first study for the molecular diagnostic of *Platynosomum* spp. in fecal sample, there is no literature reference for detection rate to compare with the current findings, thus we compared with the minimal standard of the target DNA that should be present in the sample. The minimum recommended standard for the low complexity target template such as recombinant plasmids is $10 \times 10^3 - 12 \times 10^3$ copies, while for higher complexity target template such as human gDNA is 25 – 100 ng for every 25 µl of PCR reaction (Iqbal, Junaid, 2014). Therefore, as a consequence of the present of LoD results of cPCR and TaqMan real-time PCR techniques indicated that it is great efficient and effective to apply for detection of *Platynosomum* spp. egg DNA in fecal sample.

The most common used for distinguishing the previously identified species that have similar morphological features are mitochondrial DNA (mtDNA) and ITS in molecular parasitology (Morgan and Blair, 1995; Kane et al., 1996; Morgan and Blair, 1998; León-Règagnon et al., 1999; Tkach et al., 2000; Scholz et al., 2004). The divergence percentage of the congener pairs in platyhelminths are 10% at mtDNA and 1% at ITS regions. This degree of variation makes ITS regions as a typical useful genetic marker for elucidating the intraspecific and interspecific closely related species with lesser intra-individual variations among the same species than mtDNA (Vilas et al., 2005). Moreover, the most availability of reference sequences for *Platynosomum* spp. is ITS1 regions rather than other genetic markers in GenBank. Its driving force led to pick the ITS1 region for primer design in the current study. However, the evaluation of the molecular diagnostic detection methods with more genetic markers may vary specificity, sensitivity and LoD.

The overall number of positive samples obtained by cPCR was 45.8%, which was lower than the gold standard (50%) and demonstrated moderate agreement between to methods, according to Kappa analysis. As a result, it may be assumed that microscopy and cPCR identify *Platynosomum* spp. eggs in feces at equal rates. When comparing to the gold standard, the sensitivity and PPV of cPCR are 80% and 87.27%. It is indicated that the cPCR's has 80% chance of correctly identifying all the

cats that do indeed have *Platynosomum* infection from among cats that are known to have infection, and 87.27% chance of correctly identifying all cats that do actually have the infection from among cats that might or might not have the infection. The LR+ value for cPCR technique is 6.86 (> 1), while LR- value is 0.23 (< 1). According to Edman and Runge, 2014, the probability magnitude of the cPCR technique's performance is moderate for detecting positive and negative infection. It is indicated that this method is useful for detecting the *Platynosomum* spp. eggs in cat feces.

The total positive samples of TaqMan real-time PCR technique is 55%, which is greater than the gold standard. Based on the sensitivity and PPV of TaqMan real-time PCR technique, this technique has a probability of 98.33% for detecting correct positive results in infected cats and 89.39% for distinguishing the infected cats from non-infected cats. This indicates that this method has a high level of performance. For TaqMan real-time PCR technique, the LR+ value is 8.43 (> 1) and LR- value is 0.02 (< 1). LR+ is fairly high and close to 10 and LR- is quite low and less than 1 (Edman and Runge, 2014). It suggests that using TaqMan real-time PCR to detect the *Platynosomum* spp. eggs in cat feces is very useful.

Surprisingly, cPCR and TaqMan real-time PCR revealed seven positive samples out of sixty microscopically negative samples. According to the history of the samples that used in the current study, the total number of sixty microscopically negative samples were composed of thirty samples from the cats that are known to have *Platynosomum* spp. infection and thirty samples from the cats that might or might not have this parasite infection. Since *Platynosomum* spp. shed the eggs inconsistently and has relatively low egg shedding rate (Basu and Charles, 2014), this may lead to false negative from microscopic screening and/or sub-sampling error and/or interpreter's error (Hunt and Lello, 2012). Therefore, seven positive samples that emerged by using cPCR and TaqMan methods could not possibly be considered as false positives despite the microscopic examination was the 'gold standard' to compare in this study.

Because this study is the first preliminary screening for application of PCR methods in the diagnosis of *Platynosomum* spp. infection in cats and their performance is unknown, microscopic examination was determined as the 'gold

standard' to compare in this study. As a result, true positives and true negatives were determined based on the findings of microscopic examination. Unless the 'gold standard' determination is made, it can be assumed that the PCR-based methods established in this study were more sensitivity than the current microscopic examination. As indicating in the result that PCR techniques can detect the *Platynosomum* spp. gDNA and standard plasmids at the concentrations as low as 0.1 ng/µl and 10 copies/µl for cPCR and 0.01 ng/µl and 10 copies/µl for TaqMan real-time PCR, respectively. This implies that both PCR-based methods have a high sensitivity for detecting *Platynosomum* infection in cat fecal samples.

These facts made the way to consider the containment of the parasite eggs in examined samples. *Platynosomum* spp. is a hepatic trematode and their egg production rate is 2 – 10 eggs per day in lower parasite burden and 10 – 100 eggs per day in higher parasite burdens. Hence, it is assumed that the presence of the eggs in feces is relative low (Basu and Charles, 2014). In this study, the examined samples were taken from the leftover sediments that had been previously subtracted for microscopic examination. Therefore, as a result, the chance of finding an egg in the remaining feces samples are likely to be inconsistent.

Twelve out of sixty microscopically positive fecal samples were negative in the cPCR method, despite displaying positive in TaqMan real-time PCR method. When compared to cPCR method (0.1 ng/µl), the LoD of TaqMan real-time PCR method was 0.01 ng/µl, indicating better sensitivity. This can be based on the assumption that the concentration of twelve fecal samples is lower than the cPCR limit of detection. On the other hand, it is possible that the very low amount of amplicons detected by UV gel electrophoresis reading is not visible to the naked eyes, whereas TaqMan real-time PCR reading is achieved by automatic machine. This suggests that TaqMan real-time PCR method has stronger discriminative power and sensitivity than cPCR method.

The current study is preliminary qualitative screening for the use of cPCR and TaqMan real-time PCR methods for diagnosing *Platynosomum* spp. eggs in cats feces. The study provided information on the sensitivity and specificity of the cPCR and TaqMan real-time PCR methods used in this study. Due to the limitation of the

information and genetic markers of *Platynosomum* spp. in literature, the current study relied solely on the ITS1-5.8S region as a diagnostic marker. Although ITS region has the universality, availability and PCR consistency (Yang and Hong, 2018), ITS alone is not sufficient to identify all *Platynosomum* isolates at the species level. Therefore, if different genetic markers are employed for further investigation, it may be possible to provide additional information about the molecular approach to *Platynosomum* spp. and improve data reliability and reproducibility. Nevertheless, the current study indicated that the TaqMan real-time PCR method has a high level of sensitivity and specificity. In the future, TaqMan real-time PCR method should be used for detection of *Platynosomum* spp. infection in cat, and it should be applied in combination with standard fecal egg count number for estimation of worm intensity, severity prediction and development of treatment in pre-test and post-test trial investigation.

Conclusions

According to the finding, TaqMan real-time PCR method has a better sensitivity than the cPCR method for detection of *Platynosomum* infection via cat feces. Furthermore, TaqMan real-time PCR method can assess a higher prevalence than the gold standard method. As a result, TaqMan real-time PCR method performed better than the cPCR and gold standard methods. This suggests that the TaqMan real-time PCR method developed in this study can be used for detection of *Platynosomum* spp. infections in cat.

Recommendations

To identify *Platynosomum* spp. infection in cats, the current investigation relied on the detection of egg in the examined fecal samples. In the case of complete biliary obstruction and at the outset of infection, the eggs may be absent in the feces (Basu and Charles, 2014), therefore, diagnostic methods that based on coprological samples cannot be used. As a result, other diagnostic techniques such

as immuno-diagnostic tests, ultrasonography and other imaging techniques should be examined as an alternative method for diagnosing the *Platynosomum* infections in cats.

REFERENCES

REFERENCES

Acharya KR, Dhand NK, Whittington RJ and Plain KM 2017. PCR inhibition of a quantitative PCR for detection of *Mycobacterium avium* subspecies Paratuberculosis DNA in feces: diagnostic implications and potential solutions. Front. Microbiol. 8: 115.

Adisakwattana P, Yoonuan T, Phuphisut O, Poodeepiyasawat A, Homsuwan N, Gordon CA, McManus DP, Atkinson LE, Mousley A and Gobert GN 2020. Clinical helminthiases in Thailand border regions show elevated prevalence levels using qPCR diagnostics combined with traditional microscopic methods. Parasit Vectors. 13(1): 416.

Aklakur M, Asharf Rather M and Kumar N 2016. Nanodelivery: an emerging avenue for nutraceuticals and drug delivery. Critical reviews in food science and nutrition. 56(14): 2352-2361.

Altman DG 1990. Practical statistics for medical research. In: CRC press.

Andrade RLFS, Dantas AFM, Pimentel LA, Galiza GJN, Carvalho FKL, Costa VMM and Riet-Correa F 2012. *Platynosomum fastosum*-induced cholangiocarcinomas in cats. Vet. Parasitol. 190(1-2): 277-280.

Arceo FG, Alvarado MS and Cruz RdJ 1999. Platinosomiasis en animales: una revisión. Revista Científica de la Facultad de Ciencias Veterinarias. 9(1): 60-71.

Ash LR 1962. Helminth parasites of dogs and cats in Hawaii. J. Parasitol. 48(1): 63-65.

Ayana M, Cools P, Mekonnen Z, Biruksew A, Dana D, Rashwan N, Prichard R, Vlaminck J, Verweij JJ and Levecke B 2019. Comparison of four DNA extraction and three preservation protocols for the molecular detection and quantification of soil-transmitted helminths in stool. PLoS Negl Trop Dis. 13(10): e0007778.

Barriga O, Caputo C and Weisbrode S 1981. Liver flukes (*Platynosomum concinnum*) in an Ohio cat. J. Am. Vet. Med. Assoc. 179, 901–903

Basu A and Charles R 2014. A review of the cat liver fluke *Platynosomum fastosum* Kossack, 1910 (Trematoda: Dicrocoeliidae). Vet. Parasitol. 200(1-2): 1-7.

Basuni M, Muhi J, Othman N, Verweij JJ, Ahmad M, Miswan N, Rahumatullah A, Aziz FA, Zainudin NS and Noordin R 2011. A pentaplex real-time polymerase chain

reaction assay for detection of four species of soil-transmitted helminths. The American journal of tropical medicine and hygiene. 84(2): 338-343.

Bielsa L and Greiner E 1985. Liver flukes (*Platynosomum concinnum*) in cats. The Journal of the American Animal Hospital Association. 21, 269–274

Bowman DD, Hendrix CM, Lindsay DS and Barr SC 2002. Feline clinical parasitology. In: Iowa State University Press, Ames. x + 469 pp.

Braga RR, Teixeira AC, Oliveira JAA and Cavalcanti LPG 2016. Prevalence of *Platynosomum fastosum* infection in free roaming cats in northeastern Brazil: Fluke burden and grading of lesions. Vet. Parasitol. 227: 20-25.

Bujang MA and Adnan TH 2016. Requirements for Minimum Sample Size for Sensitivity and Specificity Analysis. Journal of clinical and diagnostic research : JCDR. 10(10): YE01-YE06.

Cao Y, Griffith JF, Dorevitch S and Weisberg SB 2012. Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring *Enterococcus* in environmental waters. J. Applied Microbiol. 113(1): 66-75.

Carreira VS, Vieira RF, MACHADO GF and LUVIZOTTO MC 2008. Feline cholangitis/cholangiohepatitis complex secondary to *Platynosomum fastosum* infection in a cat. Revista Brasileira de Parasitologia Veterinária. 17(1): 184-187.

Cohen J 1960. A Coefficient of Agreement for Nominal Scales. Educational and Psychological Measurement. 20(1): 37-46.

Cullen JM and Stalker MJ 2016. Liver and biliary system. Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 2. 258.

Demeler J, Ramünke S, Wolken S, Ianiello D, Rinaldi L, Gahutu JB, Cringoli G, von Samson-Himmelstjerna G and Krücken J 2013. Discrimination of gastrointestinal nematode eggs from crude fecal egg preparations by inhibitor-resistant conventional and real-time PCR. PloS one. 8(4): e61285.

Edman E and Runge T 2014. Sensitivity, specificity, LR+, and LR−: What are they and how do you compute them. Indiana University of Pennsylvania: Indiana, PA,

USA. Retrieved from https://www.iup.edu/WorkArea/DownloadAsset.aspx?id=169819

Eisenbraun H, Alluwie A, Connolly S, Nair R, Verocai GG and Ketzis JK 2020. Comparison of fecal analysis methods for the detection of *Platynosomum fastosum* in naturally infected cats. J. Feline Med. Surg. 22(4): 395-398.

Emma B 2020. Global dog and cat population 2018. Retrieved from https://www.statista.com/statistics/1044386/dog-and-cat-pet-population-worldwide/

Evans J and Green P 1978. Preliminary evaluation of four anthelmintics against the cat liver fluke, *Platynosomum concinnum*. Aus. Vet. J 54(9): 454-455.

Ferreira A, Paes de Almeida E and Labarthe N 1999. Liver fluke infection (*Platynosomum concinnum*) in Brazilian cats: prevalence and pathology. Feline Practice. 27, 19–22.

Foley RH 1994. *Platynosomum concinnum* infection in cats. The Compendium on continuing education for the practicing veterinarian (USA). 16, 1271–1277

Gordon CA, Gray DJ, Gobert GN and McManus DP 2011. DNA amplification approaches for the diagnosis of key parasitic helminth infections of humans. Molecular and cellular probes. 25(4): 143-152.

Greve J and Leonard P 1966. Hepatic flukes (*Platynosomum concinnum*) in a cat from Illinois. J. Am. Vet. Med. Assoc. 149(4): 418-420.

Haney DR, Christiansen JS and Toll J 2006. Severe cholestatic liver disease secondary to liver fluke (*Platynosomum concinnum*) infection in three cats. J Am Anim Hosp Assoc. 42(3): 234-237.

Headley SA, Gillen MA, Sanches AWD and Satti MZ 2012. *Platynosomum fastosum*-induced chronic intrahepatic cholangitis and *Spirometra* spp. infections in feral cats from Grand Cayman. J. Helminthol. 86(2): 209-214.

Hillis DM and Dixon MT 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q Rev Biol. 66(4): 411-453.

Huang W, He B, Wang C and Zhu X 2004. Characterisation of Fasciola species from Mainland China by ITS-2 ribosomal DNA sequence. Vet. Parasitol. 120(1-2): 75-83.

Hunt PW and Lello J 2012. How to make DNA count: DNA-based diagnostic tools in veterinary parasitology. Vet. Parasitol. 186(1): 101-108.

Hwang UW and Kim W 1999. General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics. Korean J. Parasitol. 37(4): 215-228.

Iqbal, Junaid. (2014). Re: How much DNA template (genomic or plasmid DNA) is used for a general PCR?. Retrieved from: https://www.researchgate.net/post/How-much-DNA-template-genomic-or-plasmid-DNA-is-used-for-a-general-PCR/52e2474cd685cc282f8b457f/citation/download.

Jitsamai W, Khrutkham N, Hunprasit V, Chandrashekar R, Bowman D and Sukhumavasi W 2021. Prevalence of endoparasitic and viral infections in client-owned cats in metropolitan Bangkok, Thailand, and the risk factors associated with feline hookworm infections. Vet. Parasitol: Regional Studies and Reports. 25: 100584.

Jittapalapong S, Inparnkaew T, Pinyopanuwat N, Kengradomkij C, Sangvaranond A and Wongnakphet S 2007. Gastrointestinal parasites of stray cats in Bangkok metropolitan areas, Thailand. Katsetsart Journal of Natural Science. 41: 69-73.

Kane RA, Ridgers IL, Johnston DA and Rollinson D 1996. Repetitive sequences within the first internal transcribed spacer of ribosomal DNA in schistosomes contain a Chi-like site. Molecular Biochem. Parasitol. 75(2): 265-269.

Kim EM, Verweij JJ, Jalili A, van Lieshout L, Choi MH, Bae YM, Lim MK and Hong ST 2009. Detection of Clonorchis sinensis in stool samples using real-time PCR. Ann Trop Med Parasitol. 103(6): 513-518.

Kim JM, Che JH, Jeong DH and Kang BC 2010. *Platynosomum fastosum* infestation in a domestic cat in Korea. Lab. Ani. Res. 26(3): 307-309.

Köster LS, Shell L, Ketzis J, Rajeev S and Illanes O 2017. Diagnosis of pancreatic disease in feline platynosomosis. J. Feline Med. Surg. 19(12): 1192-1198.

Krecek R, Moura L, Lucas H and Kelly P 2010. Parasites of stray cats (*Felis domesticus* L., 1758) on St. Kitts, West Indies. Vet. Parasitol. 172(1-2): 147-149.

Landis JR and Koch GG 1977. The measurement of observer agreement for categorical data. biometrics. 159-174.

Lathroum CN, Shell L, Neuville K and Ketzis JK 2018. Efficacy of Praziquantel in the Treatment of *Platynosomum fastosum* in Cats with Natural Infections. Veterinary Sciences. 5(2): 35.

Leam G and Walker I 1963. The occurrence of *Platynosomum fastosum* in domestic cats in the Bahamas. Vet. Rec. 75(2): 46-47.

Lenis C, Navarro JF and Velez I 2009. First case of platinosomosis from Colombia: *Platynosomum illiciens* (Digenea: Dicrocoeliidae) in Felis catus, Turbo, Antioquia. Revista Colombiana de Ciencias Pecuarias. 22(4): 659-663.

León-Règagnon V, Brooks DR and de Leon GP-P 1999. Differentiation of Mexican species of *Haematoloechus* Looss, 1899 (Digenea: *Plagiorchiformes*): molecular and morphological evidence. J. Parasitol. 935-946.

Maldonado JF 1945. The life-history and biology of *Platynosomum fastosum* Kossak, 1910 (Trematoda: Dicrocoeliidae). Puerto Rico J. Public Health Trop. Med. 21(1): 17-39.

McHugh ML 2012. Interrater reliability: the kappa statistic. Biochem Med (Zagreb). 22(3): 276-282.

Mohanta UK, Ichikawa-Seki M, Hayashi K and Itagaki T 2015. Morphological and molecular characterization of *Eurytrema cladorchis* parasitizing cattle (Bos indicus) in Bangladesh. Parasitology research. 114(6): 2099-2105.

Montserin S, Munoz K, Seebaransingh R and Basu A 2013. Clinical case: *Platynosomum fastosum* Kossack, 1910 infection in a cat: first reported case in Trinidad and Tobago. Revue de Médecine Vétérinaire. 164(1): 9-12.

Morgan J and Blair D 1995. Nuclear rDNA ITS sequence variation in the trematode genus *Echinostoma*: an aid to establishing relationships within the 37-collar-spine group. Parasitology. 111(5): 609-615.

Morgan JAT and Blair D 1998. Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among *Echinostoma* species (Trematoda). Parasitol. 116(3): 289-297.

Muksombat K, Hoisang S, Watweingkam N, Papirom P, Tangkawattana S, Tesana S and Aunpromma S 2008. Prevalence of gastrointestinal parasites in cats in villages

of Chi Basin, Khon Kaen Province. Proceedings of the 9th Khon Kaen Veterinary Conference:11-12.

Mundim T, Oliveira Júnior S, Rodrigues D and Cury M 2004. Freqüência de helmintos em gatos de Uberlândia, Minas Gerais. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 56(4): 562-563.

Nguyen HM, Tatonova YV and Madsen H 2018. Infections by hepatic trematodes in cats from slaughterhouses in Vietnam. J. Parasitol. 104(3): 306-309.

Nguyen HM, Van Hoang H and Ho LT 2017. *Platynosomum fastosum* (Trematoda: Dicrocoeliidae) from cats in Vietnam: morphological redescription and molecular phylogenetics. Korean J. Parasitol. 55(1): 39.

Orosová M, Ivica K-H, Eva B and Marta Š 2010. Karyotype, chromosomal characteristics of multiple rDNA clusters and intragenomic variability of ribosomal ITS2 in *Caryophyllaeides fennica* (Cestoda). Parasitology International. 59(3): 351-357.

Palumbo N, Perri S, Loo B, Taylor D and Reece V 1974. Cat liver fluke, *Platynosomum concinnum*, in Hawaii. Am. J. Vet. Res. 35, 1455

Phuphisut O, Yoonuan T, Sanguankiat S, Chaisiri K, Maipanich W, Pubampen S, Komalamisra C and Adisakwattana P 2014. Triplex polymerase chain reaction assay for detection of major soil-transmitted helminths, *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, in fecal samples. Southeast Asian J Trop Med Public Health. 45(2): 267-275.

Pinto HA, Mati VLT and Melo AL 2016. Can the same species of *Platynosomum* (Trematoda: Dicrocoeliidae) infect both mammalian and avian hosts? J. Helminthol. 90(3): 372-376.

Pinto HA, Pulido-Murillo EA, Braga RR, Mati VLT, Melo AL and Tkach VV 2018b. DNA sequences confirm low specificity to definitive host and wide distribution of the cat pathogen *Platynosomum illiciens* (= *P. fastosum*) (Trematoda: Dicrocoeliidae). Parasitol Res. 117(6): 1975-1978.

Powell K 1970. Liver fluke infection in a cat. J. Am. Vet. Med. Assoc. 156(2).

Ramos RAN, Lima VFS, Monteiro MFM, de Andrade Santana M, Lepold R, da Gloria Faustino MA, Rinaldi L, Cringoli G and Alves LC 2016. New insights into

diagnosis of *Platynosomum fastosum* (Trematoda: Dicrocoeliidae) in cats. Parasitol Res. 115(2): 479-482.

Repetto S, Soto CA, Cazorla SI, Tayeldin ML, Cuello S, Lasala MB, Tekiel VS and Cappa SG 2013. An improved DNA isolation technique for PCR detection of *Strongyloides stercoralis* in stool samples. Acta tropica. 126(2): 110-114.

Retnasabapathy A and Prathap K 1971. The liver-fluke *Platynosomum fastosum* in domestic cats. Vet. Rec. 88(3).

Robinson V and Ehrenford F 1962. Hepatic lesions associated with liver fluke (*Platynosumum fastosum*) infection in a cat. Am. J. Vet. Res. 23(97): 1300-1303.

Rocha NO, Portela RW, Camargo SS, Souza WR, Carvalho GC and Bahiense TC 2014. Comparison of two coproparasitological techniques for the detection of *Platynosomum* spp. infection in cats. Vet. Parasitol. 204(3-4): 392-395.

Rodriguez-Vivas R, Williams J, Quijano-Novelo A, Bolio G and Torres-Acosta J 2004. Prevalence, abundance and risk factors of liver fluke (*Platynosomum concinnum*) infection in cats in Mexico. Veterinary record. 154(22): 693-693.

Rojekittikhun W, Chaisiri K, Mahittikorn A, Pubampen S, Sa-Nguankiat S, Kusolsuk T, Maipanich W, Udonsom R and Mori H 2014. Gastrointestinal parasites of dogs and cats in a refuge in Nakhon Nayok, Thailand. Southeast Asian J. Trop. Med. Public Health. 45(1): 31.

Rojekittikhun W, Mahittikorn A, Prummongkol S, Puangsa-art S, Chaisiri K and Kusolsuk T 2008. Prevalence of gastrointestinal parasitic infections in refuge dogs and cats and evaluation of two conventional examination techniques. J. Trop. Med. Parasitol. 36, 58–67.

Rojekittikhun W, Mahittikorn A, Prummongkol S, Puangsa-art S, Chaisiri K and Kusolsuk T 2015. Evaluation of Sugar Flotation and Formalin-Ether Concentration Techniques in the Examination of GI Parasites of Refuge Dogs and Cats in Kanchanaburi Province, Thailand. J. Trop. Med. Parasitol. 38: 17-24.

Rollinson D, Walker TK, Knowles R and Simpson A 1990. Identification of schistosome hybrids and larval parasites using rRNA probes. Systematic Parasitology. 15(1): 65-73.

Saad MZ, Chulan U and Wong W 1984. *Platynosomum Fastosum* Infestation in. ERTflMHA. 87.

Salomão M, Souza-Dantas LM, Mendes-de-Almeida F, Branco AS, Bastos OP, Sterman F and Labarthe N 2005. Ultrasonography in hepatobiliary evaluation of domestic cats (*Felis catus*, L., 1758) infected by *Platynosomum* Looss, 1907. Intern J Appl Res Vet Med. 3(3): 271-279.

Scholz T, Škeříková A, Shimazu T and Grygier MJ 2004. A taxonomic study of species of *Bothriocephalus Rudolph*i, 1808 (Cestoda: Pseudophyllidea) from eels in Japan: morphological and molecular evidence for the occurrence of B. claviceps (Goeze, 1782) and confirmation of the validity of *B. japonicus* Yamaguti, 1934. System. Parasitol. 57(2): 87-96.

Shanta C, Wan S and Kwong K 1980. A survey of the endo-and ectoparasites of cats in and around Ipoh, West Malaysia. Malaysian Vet. J. 7(1): 17-27.

Sitko J 1998. Trematodes of birds of prey (*Falconiformes*) in Czech Republic. Helminthologia. 35(3): 131-146.

Soldan MH and Marques SMT 2011. Platinosomose: Abordagem na clínica felina. Revista da FZVA. 18(1).

Soulsby EJL 1968. Helminths, arthropods and protozoa of domesticated animals. Helminths, arthropods and protozoa of domesticated animals. 26–27.

Taniuchi M, Verweij JJ, Noor Z, Sobuz SU, Lieshout L, Petri WA, Jr., Haque R and Houpt ER 2011. High throughput multiplex PCR and probe-based detection with Luminex beads for seven intestinal parasites. Am J Trop Med Hyg. 84(2): 332-337.

Taylor D and Perri S 1977. Experimental infection of cats with the liver fluke *Platynosomum concinnum*. Am. J. Vet. Res. 38(1): 51-54.

Ten Hove RJ, Verweij JJ, Vereecken K, Polman K, Dieye L and van Lieshout L 2008. Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal. Transactions of the Royal Society of Tropical Medicine and Hygiene. 102(2): 179-185.

Tkach VV, Pawlowski J and Sharpilo VP 2000. Molecular and morphological differentiation between species of the *Plagiorchis vespertilionis* group (Digenea, Plagiorchiidae) occurring in European bats, with a re-description of P. vespertilionis (Müller, 1780). System. Parasitol. 47(1): 9-22.

Toledo R and Fried B 2014. Digenetic trematodes. Vol 480. In: Springer. 308-309

Verweij JJ, Brienen EAT, Ziem J, Yelifari L, Polderman AM and Van Lieshout L 2007. Simultaneous Detection and Quantification of *Ancylostoma duodenale*, *Necator americanus*, and *Oesophagostomum bifurcum* in Fecal Samples Using Multiplex Real-Time PCR. Am. J. Trop. Med. Hyg. 77(4): 685-690.

Vilas R, Criscione C and Blouin M 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology. 131(6): 839-846.

Warren KS, Swan RA, Hobbs RP, Kuhn EM and Heeney JL 1998. *Platynosomum fastosum* in ex-captive orangutans from Indonesia. J. Wildlife Dise. 34(3): 644-646.

Worasith C, Kamamia C, Yakovleva A, Duenngai K, Wangboon C, Sithithaworn J, Watwiengkam N, Namwat N, Techasen A and Loilome W 2015. Advances in the diagnosis of human opisthorchiasis: development of *Opisthorchis viverrini* antigen detection in urine. PLoS neglected tropical diseases. 9(10): e0004157.

Xavier FG, Morato GS, Righi DA, Maiorka PC and Spinosa HS 2007. Cystic liver disease related to high *Platynosomum fastosum* infection in a domestic cat. J. Feline Med. Surg. 9(1): 51-55.

Yamada S, Yoshida A, Yoshida K, Kuraishi T, Hattori S, Kai C, Nagai Y, Sakoda T, Tatara M and Abe S 2012. Phylogenetic relationships of three species within the family *Heligmonellidae* (Nematoda; *Heligmosomoidea*) from Japanese rodents and a lagomorph based on the sequences of ribosomal DNA internal transcribed spacers, ITS-1 and ITS-2. Japanese J. Vet. Res. 60(1): 15-21.

Yang X and Hong C 2018. Differential usefulness of nine commonly used genetic markers for identifying *Phytophthora* species. Front. Microbiol. 9: 2334.

Zain SNM, Sahimin N, Pal P and Lewis JW 2013. Macroparasite communities in stray cat populations from urban cities in Peninsular Malaysia. Vet. Parasitol. 196(3-4): 469-477.

APPENDIX

Appendices A

Table 1. The summary of the diagnostic results using field samples subjected to the diagnostic methods in this study.

No.	Sample code	ID	Egg burden (EPG or eggs/30ul)	DNA conc: (ng/µl)	Parasite	remark	Microscope	cPCR	TaqMan real-time PCR
1	1	Suzy (2/7/20)	91.67 eggs/g	307.7	*P. fastosum*	single infection	+	+	+
2	2	Suzy (3/7/20)	15 eggs/g	391.9	*P. fastosum*	single infection	+	+	+
3	3	Suzy (4/7/20)	11 eggs/g	39.7	*P. fastosum*	single infection	+	+	+
4	4	Suzy (5/7/20)	17.5 eggs/g	353.5	*P. fastosum*	single infection	+	+	+
5	5	White (3/7/20)	19.17 eggs/g	99.7	*P. fastosum*	single infection	+	-	+
6	6	White (22/7/20)	10 eggs/g	313	*P. fastosum*	single infection	+	+	+
7	7	Ovaltine (17/7/20)	100.63 eggs/g	154.7	*P. fastosum*	single infection	+	+	+
8	8	Ovaltine (19/7/20)	206.25 eggs/g	257.2	*P. fastosum*	single infection	+	+	+
9	9	Ovaltine (20/7/20) 2nd	26.25 eggs/g	423.7	*P. fastosum*	single infection	+	-	+
10	10	Ovaltine (20/7/20) 1st	195 eggs/g	177.5	*P. fastosum*	single infection	+	+	+
11	11	Suzy (10/7/20)	12.5 eggs/g	340.8	*P. fastosum*	single infection	+	+	+
12	12	White (2/7/20)	120 eggs/g	303.2	*P. fastosum*	single infection	+	+	+
13	13	White (10/7/20)	31.25 eggs/g	402.1	*P. fastosum*	single infection	+	+	+
14	14	White (12/7/20)	45 eggs/g	456.4	*P. fastosum*	single infection	+	+	+
15	15	White (13/7/20)	31.25 eggs/g	174.4	*P. fastosum*	single infection	+	-	+
16	16	White (15/7/20)	17.5 eggs/g	402.7	*P. fastosum*	single infection	+	+	+
17	17	White (17/7/20)	15.63 eggs/g	169.5	*P. fastosum*	single infection	+	-	+
18	18	Ovaltine (21/7/20)	87.5 eggs/g	132.8	*P. fastosum*	single infection	+	+	+
19	19	Ovaltine (22/7/20)	35 eggs/g	211.7	*P. fastosum*	single infection	+	-	+

No.	Sample code	ID	Egg burden (EPG or eggs/30ul)	DNA conc: (ng/µl)	Parasite	remark	Microscope	cPCR	TaqMan real-time PCR
20	20	Ovaltine (23/7/20)	16.25 eggs/g	98.2	*P. fastosum*	single infection	+	-	+
21	21	Suzy (1/8/20)	15 eggs/g	238.5	*P. fastosum*	single infection	+	+	+
22	22	Suzy (2/8/20)	70 eggs/g	314.9	*P. fastosum*	single infection	+	+	+
23	23	Suzy (6/8/20)	17.5 eggs/g	57.8	*P. fastosum*	single infection	+	+	+
24	24	White (20/7/20)	16.88 eggs/g	201.2	*P. fastosum*	single infection	+	+	+
25	25	Ovaltine (24/7/20)	3.75 eggs/g	318.8	*P. fastosum*	single infection	+	+	+
26	26	Ovaltine (1/8/20)	8.75 eggs/g	229.3	*P. fastosum*	single infection	+	+	+
27	27	Ovaltine (4/8/20)	81.25 eggs/g	129.8	*P. fastosum*	single infection	+	+	+
28	28	Ovaltine (5/8/20)	7.5 eggs/g	206.9	*P. fastosum*	single infection	+	+	+
29	29	Bell (26/7/20)	13.75 eggs/g	361.1	*P. fastosum*	single infection	+	+	+
30	30	White (17/7/20)	15.63 eggs/g	92	*P. fastosum*	single infection	+	+	+
31	31	DD 0631	3 eggs/ 30 µl	179.2	*P. fastosum, Toxocara*	co-infection	+	-	+
32	32	DD 1509	18 eggs/ 30 µl	225.3	*P. fastosum*, Hookworm	co-infection	+	-	+
33	33	DD 1404	1 eggs/ 30 µl	59.9	*P. fastosum, Toxocara*	co-infection	+	+	+
34	34	DD 0614	1 eggs/ 30 µl	87.4	*P. fastosum, Toxocara,* Hookworm	co-infection	+	-	-
35	35	DD 0512	5 eggs/ 30 µl	94.9	*P. fastosum*, Hookworm	co-infection	+	+	+
36	36	DD 0102	5 eggs/ 30 µl	250.6	*P. fastosum, Dipylidium, Hookworm, Taenia, Toxocara, Cystoisospora*	co-infection	+	-	+
37	37	DD 0802	11 eggs/ 30 µl	35.5	*P. fastosum, Hookworm*	co-infection	+	+	+
38	38	DD 0804	5 eggs/ 30 µl	244.9	*P. fastosum, Toxocaara, Hookworm*	co-infection	+	+	+
39	39	DD 0636	44 eggs/ 30 µl	25.2	*P. fastosum, Hookworm*	co-infection	+	+	+
40	40	DD 0914	4 eggs/ 30 µl	214.7	*P. fastosum, Hookworm, Dipylidium*	co-infection	+	+	+
41	41	DD 1510	7 eggs/ 30 µl	207.7	*P. fastosum, Cystoisospora*	co-infection	+	+	+
42	42	DD 0927	3 eggs/ 30 µl	238.5	*P. fastosum, Hookworm*	co-infection	+	+	+

No.	Sample code	ID	Egg burden (EPG or eggs/30ul)	DNA conc: (ng/µl)	Parasite	remark	Microscope	cPCR	TaqMan real-time PCR
43	43	DD 1326	5 eggs/ 30 µl	326.5	*P. fastosum, Toxocara*	co-infection	+	+	+
44	44	DD 1912	1 eggs/ 30 µl	116	*P. fastosum, Hookworm*	co-infection	+	+	+
45	45	DD 2401	1 eggs/ 30 µl	368.1	*P. fastosum, Toxocara, Hookworm*	co-infection	+	+	+
46	46	DD 0319	5 eggs/ 30 µl	85.2	*P. fastosum, Toxocara, Hookworm*	co-infection	+	+	+
47	47	DD 1607	2 eggs/ 30 µl	327.6	*P. fastosum, Toxocara, Dipylidium*	co-infection	+	+	+
48	48	DD 0709	48 eggs/ 30 µl	241.5	*P. fastosum, Hookworm*	co-infection	+	+	+
49	49	DD 1402	1 eggs/ 30 µl	136.5	*P. fastosum, Toxocara, Cystoisospora*	co-infection	+	+	+
50	50	DD 1821	8 eggs/ 30 µl	97.9	*P. fastosum, Toxocara, Hookworm*	co-infection	+	+	+
51	51	DD 1005	5 eggs/ 30 µl	144	*P. fastosum, Hookworm*	co-infection	+	+	+
52	52	DD 1610	8 eggs/ 30 µl	104.2	*P. fastosum, Toxocara, Dipylidium*	co-infection	+	+	+
53	53	DD 1013	1 eggs/ 30 µl	30.1	*P. fastosum, Hookworm*	co-infection	+	-	+
54	54	DD 0504	20 eggs/ 30 µl	17.4	*P. fastosum, Hookworm*	co-infection	+	+	+
55	55	DD 1507	1 eggs/ 30 µl	29.7	*P. fastosum, Hookworm*	co-infection	+	-	+
56	56	DD 2129	7 eggs/ 30 µl	29.8	*P. fastosum, Hookworm*	co-infection	+	+	+
57	57	DD 0403	1 eggs/ 30 µl	43.4	*P. fastosum, Hookworm*	co-infection	+	+	+
58	58	DD 1920	2 eggs/ 30 µl	53.1	*P. fastosum, Hookworm*	co-infection	+	+	+
59	59	DD 0920	1 eggs/ 30 µl	30.3	*P. fastosum, Toxocara*	co-infection	+	+	+
60	2219	DD 2219	7 eggs/ 30 µl	196.8	*P. fastosum, Hookworm*	co-infection	+	+	+
61	60	DD2503	-	7.8	Hookworm, *Toxocara*	Other parasite inf:	-	+	+
62	61	DD 2512	-	417.3	Hookworm	Other parasite inf:	-	-	-
63	62	DD 2403	-	336.1	*Dipylidium, Trichuris*	Other parasite inf:	-	+	+
64	63	DD 2307	-	334.7	Hookworm, *Toxocara*	Other parasite inf:	-	+	+
65	64	DD 2521	-	232.1	Hookworm	Other parasite inf:	-	-	+
66	65	DD 2522	-	474	*Toxocara*	Other parasite inf:	-	-	-

No.	Sample code	ID	Egg burden (EPG or eggs/30ul)	DNA conc: (ng/µl)	Parasite	remark	Microscope	cPCR	TaqMan real-time PCR
67	66	DD 2323	-	135.8	*Toxocara*	Other parasite inf:	-	-	-
68	67	DD 2402	-	119.9	Hookworm	Other parasite inf:	-	-	-
69	68	DD 2409	-	344.4	Hookworm	Other parasite inf:	-	-	-
70	69	DD 2306	-	388.3	*Toxocara*	Other parasite inf:	-	-	-
71	70	DD 2316	-	186.5	Hookworm, *Toxocara*	Other parasite inf:	-	-	-
72	71	DD 2523	-	374.7	Hookworm, *Toxocara*	Other parasite inf:	-	-	-
73	72	DD 2338	-	212.5	Hookworm, *Toxocara*	Other parasite inf:	-	-	-
74	73	DD 0138	-	492.6	*Toxocara*	Other parasite inf:	-	-	-
75	74	DD 0224	-	103.1	Hookworm	Other parasite inf:	-	-	-
76	75	DD 2309	-	26.6	*Toxocara*	Other parasite inf:	-	-	-
77	76	DD 2322	-	160	Hookworm	Other parasite inf:	-	-	-
78	77	DD 2514	-	227.6	*Dipylidium*	Other parasite inf:	-	-	-
79	78	DD 2513	-	276.6	Hookworm	Other parasite inf:	-	-	-
80	79	DD2401	-	190.5	Hookworm, *Cystoisospora*	Other parasite inf:	-	-	-
81	80	DD 0205	-	102.4	*Taenia taeniaeformis*	Other parasite inf:	-	-	-
82	81	DD 2421	-	83.5	*Cystoisospora*	Other parasite inf:	-	-	-
83	82	DD 2005	-	55.1	*Taenia taeniaeformis*	Other parasite inf:	-	-	-
84	83	DD 2407	-	10.5	*Toxocara*	Other parasite inf:	-	-	-
85	84	DD 2524	-	73.8	Hookworm, *Toxocara*	Other parasite inf:	-	-	-
86	85	DD 0117	-	213.2	Cystoisospora, Dipylidium	Other parasite inf:	-	-	-
87	86	DD 0101	-	152.5	*Spirometra*, Hookworm	Other parasite inf:	-	-	-
88	87	DD 2337	-	7.9	*Toxocara, Cystoisospora*	Other parasite inf:	-	-	-
89	88	DD 2411	-	817.6	*Trichuris*	Other parasite inf:	-	-	-
90	89	DD 0206	-	180.2	*Toxocara, Taenia taeniaeformis*	Other parasite inf:	-	-	-

No.	Sample code	ID	Egg burden (EPG or eggs/30ul)	DNA conc: (ng/µl)	Parasite	remark	Microscope	cPCR	TaqMan real-time PCR
91	90	Suzi (7/7/20)	-	212.4	-	Non-parasite detected	-	-	-
92	91	Suzi (4/8/20)	-	132.6	-	Non-parasite detected	-	-	-
93	92	White (4/8/20)	-	280	-	Non-parasite detected	-	-	-
94	93	White (23/7/20)	-	273	-	Non-parasite detected	-	-	-
95	94	White (16/7/20)	-	313.3	-	Non-parasite detected	-	-	-
96	95	Beauti (5/8/20)	-	336.9	-	Non-parasite detected	-	-	-
97	96	Bell (5/8/20)	-	133	-	Non-parasite detected	-	-	-
98	97	Beauti (4/8/20)	-	466.9	-	Non-parasite detected	-	-	-
99	98	Ovaltine (2/8/20)	-	38.2	-	Non-parasite detected	-	+	+
100	99	White (6/8/20)	-	397.6	-	Non-parasite detected	-	+	-
101	100	Ovaltine (1/8/20)	-	153.6	-	Non-parasite detected	-	-	-
102	101	White (14/7/20)	-	257.6	-	Non-parasite detected	-	-	-
103	102	Beauti (20/7/20)	-	467.8	-	Non-parasite detected	-	-	-
104	103	White (4/7/20)	-	273.6	-	Non-parasite detected	-	-	-
105	104	Suzy (6/7/20)	-	273.3	-	Non-parasite detected	-	-	-
106	105	White (18/7/20)	-	272	-	Non-parasite detected	-	-	-
107	106	White (7/7/20)	-	244.7	-	Non-parasite detected	-	-	-
108	107	white (21/7/20)	-	284.5	-	Non-parasite detected	-	-	-
109	108	Beauti (28/7/20)	-	325.8	-	Non-parasite detected	-	-	-
110	109	Ovaltine (27/7/20)	-	206.1	-	Non-parasite detected	-	-	-
111	110	White (1/8/20)	-	143.8	-	Non-parasite detected	-	-	-
112	111	White (27/7/20)	-	309.6	-	Non-parasite detected	-	+	+
113	112	Beauti (19/7/20)	-	81.4	-	Non-parasite detected	-	-	-
114	113	Beauti (4/8/20)	-	103.2	-	Non-parasite detected	-	-	-

No.	Sample code	ID	Egg burden (EPG or eggs/30ul)	DNA conc: (ng/µl)	Parasite	remark	Microscope	cPCR	TaqMan real-time PCR
115	114	Beauti (22/7/20)	-	72.9	-	Non-parasite detected	-	+	+
116	115	White (6/7/20)	-	156.6	-	Non-parasite detected	-	-	-
117	116	Bell (6/8/20)	-	48.2	-	Non-parasite detected	-	-	-
118	117	Bell (28/7/20)	-	225.6	-	Non-parasite detected	-	-	-
119	118	Beauti (21/7/20)	-	90.5	-	Non-parasite detected	-	-	-
120	119	Beauti (6/8/20)	-	53.9	-	Non-parasite detected	-	-	-

Appendices B

Table 2. DNA concentration of common feline parasites used for specificity testing in this study

No.	Parasite	DNA concentration (ng/µl)
1.	*Toxocara* spp.	120.1
2	*Ancylostoma* spp.	31.1
3	*Strongyloides* spp.	182.7
4	*Taenia taeniaeformis*	164.9
5	*Dipylidium caninum*	117
6	*Spirometra* spp.	176.6
7	*Opisthorchis viverrini*	37.8
8	*Cryptosporidium* spp.	25.1
9	*Toxoplasma gondii*	37.3
10	*Giardia* spp.	27.6
11	*Cystoisospora* spp.	166.2

Appendices C

Table 3. The sequences of the amplicon derived from amplification of adult worm and egg DNA of *Platynosomum* spp. Isolated from naturally infected cats

No.	Sample	ID	Sequence
1	Adult worm DNA	P01	TGGTCTTGAGCCTCTGTTGTGGGGACGGGATGTACTGTAAAT ATTACGGTGCTAGGCTTAATGAGTGGTGAATATCAAGAGCTA CGGCTCGGCCACCGCCCTAAGTCCTCCCCATTTCACATCATGA GTGATGCATGCTGGTTTCGACCGGTGTGTGTCAATGCTCATTA ATGCTCCCGGCCTACACTGGGTTGCATTTGCAGTCGCCTGGCG TTGCCTTGACCTGGGCTTGACTGTGAAATGACTTATCACTCTGG
2	Bile egg DNA	P08	CGGYCYTGGTCTTGAGCCTCTGTTGTGGGGACGGGATGTACTG TAAATATTACGGTGCTAGGCTTAATGAGTGGTGAATATCAAGA GCTACGGCTCGGCCACCGCCCTAAGTCCTCCCCATTTCACATCA TGAGTGATGCATGCTGGTTTCGACCGGTGTGTGTCAATGCTCAT TAATGCTCCCGGCCTACACTGGGTTGCATTTGCAGTCGCCTGGC GTTGCCTTGACCTGGGCTTGACTGTGAAATGACTTATCACTCTGG
3	Fecal egg DNA	P23	CTCAGGCCTTGGTCTTGAGCCTCTGTTGTGGGGAGGGATGTACT GTAAATATTACGGTGCTAGGCTTAATGAGTGGTGAATATCAAGA GCTACGGCTCGGCCACCGCCCTAAGTCCTCCCCATTTCACATCAT GAGTGATGCATGCTGGTTTCGACCGGTGTGTGTCAATGCTCATTA ATGCTCCCGGCCTACACTGGGTTG

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